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TISSUE CULTURE

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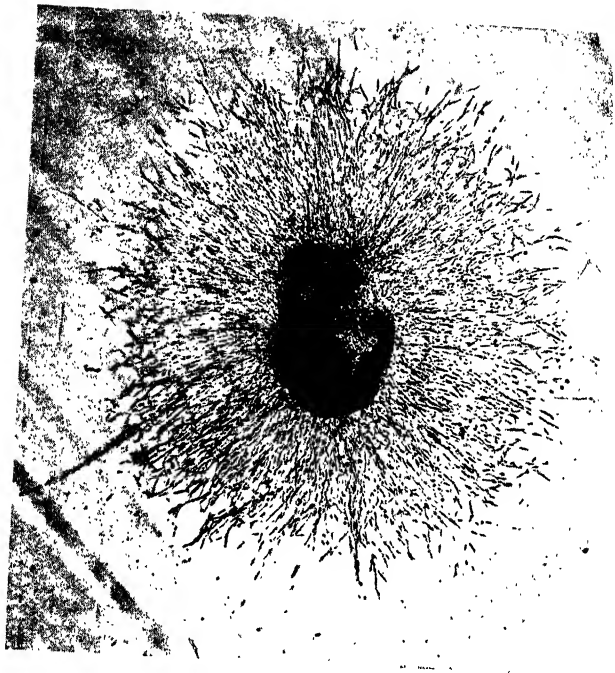

Fig. 1—Colony of perichondral fibroblasts, forty-eight hours after explantation

The original fragment shows as a dark area in the centre, while the cells forming the new growth are spreading out in the medium around it. (*From a photograph kindly supplied by Dr. F. Jacoby.*)

TISSUE CULTURE

THE GROWTH AND DIFFERENTIATION OF NORMAL TISSUES IN ARTIFICIAL MEDIA

BY

E. N. WILLMER, M.A.

PHYSIOLOGICAL LABORATORY
CAMBRIDGE

WITH 2 PLATES AND 9 TEXT DIAGRAMS

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PREFACE

TISSUE culture has in the past been the object of much misrepresentation and of many false conceptions. By some, optimists, it has been regarded as a magic key to the understanding of the life process; by others, pessimists, it has been slighted as an almost valueless craft which has contributed nothing to science. Needless to say, neither of these points of view is in the least justified. In this book an attempt is made to give as fair an account as possible of the part played by the method of tissue culture, for it must not be regarded as anything more than a method, in helping to elucidate some of the problems of normal growth and differentiation, and in furthering the knowledge of the processes involved in the normal development of the animal organism. No attempt has been made to give a full account of all the ways in which the method of tissue culture has been applied, which would indeed be a task far beyond the compass of this book, and no treatment will therefore be found of such subjects as the growth of malignant cells, of the relative sensitivity of these and normal cells to such external influences as radium and X-rays, nor of any of the applications of the method to problems of pathology.

Those who are engaged in researches in which the cultivation of tissues in artificial media outside the body plays a prominent part are conscious of a great change in outlook with regard to the conceptions of

cellular structure and function which has been brought about by such a close study of the living cells as is possible by tissue-culture methods. The author has tried in this book to convey something of this more vital outlook to those interested in general problems of biological science, as well as to give to those working in the more restricted fields of tissue culture a summary account of the main advances which have been made in the knowledge of growth and differentiation processes, and for which the method has been responsible.

The author wishes to express his thanks to Dr. F. Jacoby, Dr. H. B. Fell and Dr. F. G. Spear for helpful criticism, and to Mr. and Mrs. H. G. Willmer for their assistance in the preparation of the manuscript.

E. N. WILLMER

PHYSIOLOGICAL LABORATORY
CAMBRIDGE
April, 1935

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INTRODUCTION

HORTICULTURE dates back to the earliest days of human achievement, and two of its essential practices must be almost equally ancient, namely the striking of cuttings and the making of successful grafts. In the light of this it is perhaps a little surprising that the successful application of such methods to animals should have been so long delayed, although naturally the results might have been expected to be different. But the reasons for this delay are not far to seek. For the success of the cutting or graft certain requirements are made both as to the character of the scion or stock and as to the nature of the soil in which it is to be placed, and for experiments of the same kind on animals a far more rigorous set of conditions is necessary.

The evolution of the higher animals has largely been the evolution of a constant internal environment, so that although the animal as a whole may be subjected to a great variety of external conditions, yet its individual cells remain in much more constant surroundings. The cells so sheltered from the chances and changes of the outside world can then specialize in the performance of their functions, and develop physico-chemical processes and reactions which depend on a finely balanced set of conditions, and which would be completely impossible in cells not so carefully protected. One result of this specialization is that the cell groups tend to lose some of their independence for the good of the whole. A parallel may be drawn from human experience. In the small hamlet the inhabitants can mostly satisfy their needs by their own efforts and from the village shop, but in the more highly organized society the draper

learns to rely on the milkman, the grocer on the butcher, and each upon the other, so that the community is dependent on the behaviour of all its members, on the railway, on the district council, and on the Government. So in the body, the skin, the stomach, the liver, and the intestine could none of them function without the other, without the blood-stream, the peripheral nervous system or the brain. In recent years the problem of unemployment has only made it too obvious that when any given member of a highly organized human community is rooted up and transferred to an environment in which he has to fend for himself, he is seldom fitted for the conditions, and in the absence of help from others must needs perish. So it is that isolated groups of cells from the animal body, unless transferred to conditions extremely similar to those to which they are accustomed, very soon languish and die. The cells of plants are far less irreversibly specialized, and so are capable of life under much simpler conditions, but with animals it is different and as knowledge of their physiology advances it becomes more and more evident that the success of the animal body depends on this proper balance of activity among its various parts, and the functioning of one organ becomes controlled by the conditions set up by another. The glycogen content of the liver, for example, is subject to the behaviour of the adrenal glands, the pituitary, and the pancreas; the respiratory movements are governed by the nature and amount of blood flowing in the carotid artery. It is not however necessary to enumerate further such examples of the balance of one system against another in the make-up of the higher animals. Among lower organisms the process has not proceeded so far. The internal environment cannot be maintained so constant, and therefore such delicate interrelationships are not possible. The cells cannot as a general rule become so specialized, and they must be able to carry out their functions in an environment in which greater or smaller changes are of constant occurrence. In short, the cells of lower organisms are

less dependent on the constancy of an internal environment than are those of higher animals.

This relative independence renders it a comparatively easy matter to make grafts and to take cuttings of such lowly organisms as the flat-worms or planarians, in much the same way as is possible with plants. Their cells are as yet for the most part not so reliant on the other cells of the body, and are capable of much greater variation and adaptability in their behaviour. It is the same story as is told again and again in the history of various forms of parasitism. In becoming a parasite the free-living independent animal sacrifices more or less of its independence to gain a relatively constant set of conditions, under which it can devote itself more thoroughly to the reproduction of its species. In higher animals the individual cells are as it were parasitic on all the other cells of the body, and, by becoming thus subservient, they can function more efficiently in one particular direction. Just as a parasite becomes helpless when removed from its position on the host, so the isolated cells of higher animals are relatively incapable of independent existence when removed from their original situations. This "parasitism" of the cells of the higher animals on one another is clearly responsible for the chief difficulty in making successful cultures of animal tissues away from their normal position. Lower animals and their cells avoid harmful environments by the method of escape, by surrounding themselves with impermeable membranes, &c. In higher animals the cells are by no means independent of their immediate environment but nevertheless can function properly because that environment has been specialized and made constant for them. To make cuttings of plants, all that is necessary is to remove part of the plant and place it in a fresh environment, and, given the necessary salts, water and light, certain of its cells will form new roots, new shoots or what-not, so that in time a whole new plant will be formed. The same operation may be performed with certain lower animals, but the more highly organized the animal the more difficult

does the operation become; the cells have lost their independence, and in experiments on higher animals success is only possible when the cells are removed to an environment very closely similar to their normal one and with which they can quickly establish equilibrium. Moreover, except in animals very low in the evolutionary scale, a whole animal can never be regenerated from a part.

An intimate knowledge then of the make-up of the normal environment of the cells of an organism was a prerequisite to successful tissue culture. With the advance of physiological knowledge, enough is now known of the immediate surroundings of tissue cells within the body to allow such cells to be cultured and to grow for long periods of time in artificial surroundings outside the parent organism. There is still, however, a very long road to be travelled before it will be possible to produce a synthetic environment for tissue-culture cells. In other words although it is possible to maintain cells alive, actively functioning and growing, outside the organism, it is still far from possible to decide exactly what are the particular necessities in their environment; but tissue culture itself should help to thread the labyrinth of such a problem.

Besides the difficulty of finding a suitable environment for the cells, a factor which delayed the beginnings of tissue culture was a lack of understanding of bacterial organisms and the consequent difficulty of establishing a suitable aseptic technique. The animal body as a whole has a variety of protective mechanisms against bacteria, &c., among which may be mentioned as an example the whole leucocyte system, by which the colourless and actively mobile cells of the blood and tissue fluids ingest and remove any foreign particles and bacteria, which may have penetrated below the skin or through the mucous membrane of the alimentary canal. These cells, although they may be found nearly all over the body, are essentially connected with the blood-stream, and are only manufactured in certain definite regions such as the lymph glands and the bone

marrow. Tissue deprived of its blood-stream is, among other things, deprived of any fresh supply of leucocytes, and hence, in this direction at least, is practically defenceless against bacteria. Media in which tissue cultures will grow or survive are media *par excellence* for the development of micro-organisms of various kinds, pathogenic and non-pathogenic, so that any slight infection of the medium of a tissue culture is almost certain, sooner or later, to prove fatal to the cells of the culture. Sometimes death may be due to the development of harmful metabolites and toxins, at other times it is on account of the exhaustion of the nutritional elements of the medium by the extremely rapid multiplication of the micro-organisms. It is thus obvious that a very rigid aseptic technique is a *sine qua non* for successful explantation experiments, and until the underlying principles of such a technique were firmly established, tissue culture was unlikely to be successful.

The first definite advances were made during the last century when it was established that balanced salt solutions, containing sodium, potassium and calcium ions in certain specified proportions were necessary for the regular pulsation of the isolated frog's heart; but it was then a long time before Harrison in 1907 [103] first successfully maintained frog nerve tissue alive and active in a hanging drop of frog lymph. This established the fact that, given suitable conditions, small groups of animal cells could function away from their position in the living animal, and it was with these experiments that tissue culture began.

It is now possible to maintain cells alive outside the organism for an apparently indefinite time, to examine them in the living condition under the highest powers of the microscope, to perform experiments upon them and, in general, to study their behaviour under a variety of conditions. This range of conditions is not, however, as great as might be desired owing to the very definite limitations which are imposed by the techniques so far developed. It is not surprising, however, now

that the fundamental requirements for tissue culture have been established, that the method should find applications in several biological fields, and indeed there is hardly a branch of biological science which has not taken advantage of the possibilities afforded by the new technique. Physiology and anatomy have perhaps reaped the greatest harvest, and particularly where they meet on the common ground of embryology. In the ensuing pages the plough of tissue culture is followed in its rather uncertain and erratic course across the field of physiology. Two particular aspects will be reviewed: one, unorganized growth, concerned with the survival and proliferation of cells, and the other, organized growth, dealing with the differentiation of growing tissues and the development of their functional capabilities.

It is both fortunate and unfortunate that the domestic fowl is a prolific bird, and that its embryo develops in a shell, isolated from the contaminations of the outside world. It is fortunate because in the chick embryo are present active living cells in various stages of development and practically free from possible infections, cells therefore which are in a state highly suitable for many kinds of tissue culture experiment. It is unfortunate in that, as a direct consequence of this, by far the greatest amount of work has been done on avian cells in an embryonic condition and there is a tendency, sometimes justifiable sometimes not, to generalize from results obtained on such tissue and to infer similar behaviour for adult or even mammalian tissue.

In the development of the chick as indeed of all other animals there are three main stages. Its cells first increase in number, then they become specialized for the performance of their particular functions, in other words they differentiate, and finally they function. What therefore may be expected of embryonic cells and tissues when removed from their natural habitat to cultural conditions outside the body? At first the main interest of tissue culture centred round the fact that such cells from chicks and embryonic

animals in general would remain alive and multiply in artificial conditions; latterly the centre of attraction in the picture has shifted, and nowadays it is chiefly the problems of differentiation and function which are receiving attention. With improved technique and greater understanding of cellular behaviour it is possible to some extent to control the activities of cells and tissues when removed into artificial conditions, and to determine whether on the one hand they shall grow and divide, or whether on the other they shall remain relatively quiescent, differentiate and even in some cases start to function. These two main types of behaviour have been classified as unorganized and organized growth, and it is on those lines that tissue culture methods have had most to contribute to physiological knowledge; but much has also been learned from direct observation and experiment on tissues which are simply in a state of survival in artificial media, and in this connexion it is perhaps unfortunate that embryonic cells have played such a prominent part.

Adult tissues can be cultured, but the results are not so spectacular; the cells do not grow readily and the tissue tends to remain relatively quiescent. There is however undoubtedly much to be gained by the study of the behaviour of isolated adult tissues, though up to the present time little has been achieved in this direction.

There is also another field to which tissue culture has much of importance to contribute, namely the study of malignant cells, and both carcinoma and sarcoma tissues of various types have been grown in vitro. The requirements of such tissues are rather special, being often quite different from those of normal cells, and indeed the presence of the latter either dead or alive is sometimes necessary for the successful culture of malignant cells. The growth rates are quite different; the tissues show many peculiarities of metabolism, and although it is probably impossible to point to any one particular histological distinction between normal and malignant cells which is always valid, yet they form a

class of tissues by themselves, and offer their own peculiar problems. For these reasons it is impossible to discuss their behaviour in the pages that follow, and attention therefore is restricted simply to problems set by normal tissues, first in connexion with their growth, and secondly those concerned with the organization of such growing tissue and the acquirement of its various functions.

UNORGANIZED GROWTH

1. CULTURE METHODS

(*a*) *Hanging-drop Technique*

TISSUES when removed from the animal and placed in suitable media such as blood plasma, serum, lymph or physiological salt solutions, such as those known as Ringer-Locke, Tyrode, or Pannet and Compton solutions, display varying degrees of activity.

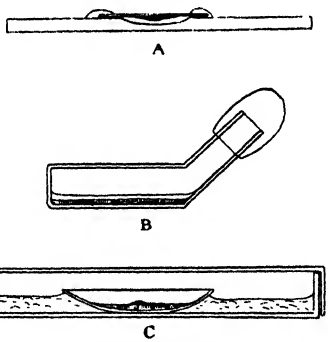

FIG. 2.—Diagram showing the three typical methods of culturing tissues in vitro. In each case the culture vessel is seen in vertical section

A, hanging drop or coverslip culture; B, flask culture; C, watch glass culture. The tissue is shown black, and the medium with vertical shading. In the flask method there is fluid above the solid coagulum.

After the tissues are placed in the medium, sooner or later, depending chiefly on the age of the tissue and the character of the medium, there is an outwandering of cells on to any surface which is available, so that in successful cultures after a day or so there is a halo of transparent new growth around the central implant,

which itself tends to become thinner and more spread out. In the simplest technique, a fragment of tissue, generally less than one millimetre in diameter, is placed in a drop of medium on a coverslip, which is then inverted over a hollow-ground microscope slide and sealed off with vaseline or paraffin-wax before being placed in an incubator to maintain the tissue at the normal temperature of the animal's body. If the medium contains plasma, it will probably set to a solid jelly. This is the standard hanging-drop technique and has perhaps been the most widely used, although it is beset with many serious limitations. The amount of medium is small, and any slight evaporation alters the concentration of its constituents very materially. Metabolites cannot escape and therefore concentrate rather rapidly in the medium, and the supply of available oxygen and foodstuffs is extremely limited. On the other hand, the cells wandering out on the surface of the coverslip, or in the meshes of the plasma coagulum, are in a situation which is ideal for microscopical examination. Generally speaking, only very small fragments of tissue can be cultured in vitro, for under such conditions the tissues may be compared to those lower animals which have not developed a blood-vascular system. Oxygen can only penetrate slowly through the living tissue and it gets used up as it goes. Likewise, metabolites have difficulty in diffusing away. Consequently the cells in the centre of the fragment sooner or later begin to suffer from oxygen lack, starvation or intoxication and may become necrotic. For this, and for the reasons outlined above, cultures in hanging-drop preparations can only be maintained in a healthy state for a very few days. In practice, the difficulties are to some extent overcome by subculturing the tissues at frequent intervals into fresh medium. Cultures may sometimes remain active though unchanged for many days, but in general the limitations of the medium become reflected in the behaviour of the cells after as short a time as two days or even thirty-six hours.

(*b*) *Flask Technique*

By another method, known as the flask technique, many of the drawbacks of the hanging-drop method are eliminated, but at the same time others are introduced. By this technique the tissues are grown in a solid coagulum of blood plasma about one millimetre in depth on the bottom of a small flask, three to five centimetres in diameter, which is flat at the top and bottom and has a neck pointing obliquely upwards attached to one side. Such flasks are known as Carrel flasks after their inventor.[31] A considerably greater quantity of medium can be employed than in hanging-drops, and the solid coagulum can be repeatedly washed and bathed by a fluid phase so that the necessity for sub-culturing the original tissue at such frequent intervals is lessened. In practice the fluid medium is probably changed two or three times per week, and the tissues transferred to a fresh flask about every ten days. These times may of course vary according to the nature of the experiment and the character of the fluid medium. The general behaviour of the tissues in cultures in flasks, though differing in detail, is essentially similar to that observed in hanging-drop cultures in a solid medium. There are various modifications of the flask technique adapted for special purposes. Its main drawback is that the cells are not in a convenient position for direct observation, but this can be overcome to some extent by sealing coverslips over holes in the top and bottom, or planting the tissues on coverslips inside the flasks, and then removing them for fixation and staining at the end of the experiment. By the use of coverslips sealed to the bottom of flasks with pure shellac, and with the aid of a specially inverted microscope [199] it is possible to examine tissues under quite high magnification as they grow undisturbed in a plasma coagulum for many days.

(*c*) *Watch-glass Technique*

—A third method which is frequently employed is to set the tissues either in or on a plasma coagulum in a

watch-glass and place the whole in a Petrie dish in which moist sterile cotton-wool prevents evaporation from the medium.[80] This and closely similar techniques have been employed with conspicuous success in studies on organized growth and differentiation. Various other slight modifications of the three methods described have been used for attacking special problems, but these three remain the standard types and will be referred to as the hanging-drop, flask and watch-glass techniques, respectively.

2. CHARACTERISTICS OF GROWTH

When a fragment of tissue, say of embryonic chick heart, is placed in a hanging-drop culture, in physiological salt solution, or better, in a plasma coagulum, new cells make their way out into the medium and the tissue is colloquially said to grow. This process, though apparently simple and straightforward, is in reality extremely complex and technically not really true growth. There is practically no increase in the actual mass of the tissue explanted or in the number of cells present, and attempts to subculture the tissue do not help matters, but rather the reverse. Most of the outgrowth is caused by cells actively emigrating from the centre. If to the plasma coagulum a drop of a saline extract of embryonic tissues, for convenience known as embryo extract, is added, more rapid and spectacular changes occur, cell divisions are frequently observed and the tissue quickly increases in mass; in fact, actual growth clearly takes place.

It is a pity that the term growth has been loosely applied both to the general activity of cells in culture, quite apart from any consideration as to whether the actual mass of the tissues is increasing or whether cell multiplication is occurring or not, as well as to describe the more restricted condition in which both processes are taking place; but, if the distinct uses of the term are kept in mind no confusion need occur.

(*a*) *Growth of Fibroblasts*

In the first place, if the medium be Tyrode solution, a culture of chick heart tissue shows signs of life for several days. The original fragment tends to flatten out and the cells form a protoplasmic network by

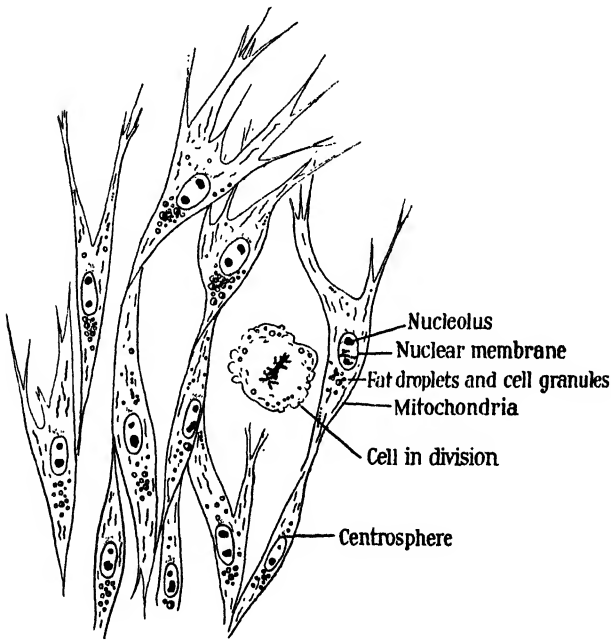

FIG. 3.—Diagrammatic representation of fibroblasts growing near the periphery of a culture

spreading out on the glass surface in a roughly centrifugal direction. The extent of this visible activity depends (among other things such as the degree of contact made between the tissue and the glass) on the age of the chick from which the tissue was taken. In general, the metabolism and growth of a tissue falls

off as the tissue ages,[165] so that as an approximate rule the younger the tissue the more activity will it display in culture. Its behaviour in Tyrode solution, which contains no food substances except glucose, or indeed in any other non-growth-promoting medium, may be taken to indicate its inherent activity, or, as it is often called, its residual growth energy.[32, 34, 70, 74] In addition to the cells which creep along the glass surface, a few may also spread along the surface between the fluid medium and the air. By far the greatest part of this so-called growth of the culture is in reality due to nothing more than the flattening of the cells and to their outward migration, so that the total number of cells at the end may be scarcely any larger than the number present in the initial implant. The cells which grow out appear more or less histologically undifferentiated and of an embryonic type. Probably they are a mixed collection of mesenchyme cells, and, collectively and for convenience, they are termed fibroblasts. The use of this term arose not because the cells necessarily have any connexion with the formation of fibres, although some of them may show this property, but because they are similar in appearance to the cells in the body which are believed to function in this manner. Their actual nature is uncertain, but meanwhile the term is used to designate a whole group of cells which grow in vitro after the fashion here described, namely forming a cellular network. This network may include cells of different types which become indistinguishable in cultural conditions; for example, heart muscle cells and endothelial cells may be present. Under these simple conditions of culture the cells have for the most part lost many of the details of structure which they possessed in the original tissue, but nevertheless there is evidence that they are not completely dedifferentiated, that is to say, they have not completely reverted to an indifferent type, and some at least are capable, given the right set of conditions, of producing fully differentiated cells, which may even be capable of function. Needless to say, the appropriate conditions seldom, if

ever, occur in such a simple medium as Tyrode solution. Typical fibroblastic growth is shown in Fig. 3. The cells near the central implant are for the most part spindle-shaped and lie with their long axes radial to the culture. Nearer the periphery they may lose this orientation to some extent and often become triangular or irregular in outline, thus illustrating one of the more important conceptions to which tissue culture has led, namely that it is extremely difficult, if not impossible, to classify cells according to their morphological appearance alone. As the spindle-shaped typical fibroblast reaches the periphery of the growth zone it may be seen to alter its whole appearance. There are also many other examples which will be discussed later where cells can be shown to owe their morphological characteristics to their surroundings. Fibroblasts move with a curious gliding motion as the cytoplasm flows towards the advancing edge of the cell. When the triangular shape has been assumed the cells move with the apex at the stern. The cytoplasm in young healthy cultures is clear and hyaline, no foam-like or alveolar structure being visible by direct illumination or by the use of the dark field.[181] There is a slight modification of the cytoplasm in the region of the nucleus, and generally on the side of the cell which lies away from the central implant, thus indicating the presence of a certain polarity in the cell. This specialized protoplasm has received several names, of which centrosphere is the most generally accepted. Its significance is obscure, but often in the same region, by vital staining with neutral red, there can be observed several small red granules which are probably connected with the Golgi apparatus. The polarity of the cell is also indicated by the position of the fat drops when they occur in the cytoplasm, for they generally collect first on the side of the cell nearest to the central implant.[156] By dark-field illumination small glistening filamentous bodies are seen moving freely through the cytoplasm of the cell in a manner which is apparently independent of cytoplasmic currents. These bodies are also rendered

clearly visible by the use of vital dyes, particularly Janus green B (Hoechst), and are known as mitochondria. The speculations as to their function are numerous, but exact knowledge is nil. From the behaviour of the Janus green under different conditions of oxygenation of the cell, it has been suggested that they provide surfaces upon which cell oxidations can occur,[119] while workers in other fields have even suggested that they may be the seat of protein synthesis. When the cells become unhealthy or damaged these rod-like bodies quickly round off and become globular,[46] while in healthy cells they can sometimes be seen to break into two by transverse splits.[181] The cell nucleus appears as an oval translucent area near the centre of the cell within which one or more globules or nucleoli may often be distinguished by their different refractive index. In most cultures there are to be seen in the cells numerous small fat droplets, and also, as mentioned above, granules or vacuoles which stain with neutral red. Both these structures are to some extent adventitious and depend on the conditions of the cell. Prolonged staining with neutral red causes the dye to penetrate freely into the cell and to be segregated into vacuoles which are probably quite different in character from the small granules which appear stained first.[48, 50] Neutral red vacuoles also appear in cultures when the medium is deficient in glucose but disappear as soon as the medium is altered.[135] When cultures are fixed and stained by impregnation methods to show the Golgi apparatus, it is found that as the cells spread away from the central implant and flatten against the glass surface, so the Golgi network breaks up [149] and becomes scattered through the cytoplasm. In fatty and degenerate cells the Golgi apparatus is often completely broken up, and some of the fat globules under these conditions owe their origin to the disrupted Golgi body. The break-up of the structure as the cells spread out on the coverslip suggests that perhaps the cells are no longer to be regarded as perfectly normal, and this illustrates what is probably a general principle applicable

to all tissue cultures—namely that the cells composing them are still living units which are in some degree capable of adapting themselves to their new surroundings and thereby becoming correspondingly modified from normal cells of the same type. This consideration applies not only to morphological characteristics, but probably with equal force to those which are more physiological. The living cell is a system which is always tending to reach equilibrium with its surroundings, and therefore is extremely dependent upon them. A cell which has been placed in a tissue culture for even a few hours will not necessarily have the same characteristics as it had in the animal from which it was removed.

(*b*) *Growth of Epithelia*

Besides fibroblasts, two other main groups of cells may be distinguished by their behaviour in vitro. Early in the development of animals, some factor or factors must determine that certain cells shall form supporting structures and the general mesenchymal cells of the adult, whilst something else determines that other cells shall become epithelia and cover the surfaces of the body, for, in vitro, certain cells habitually grow as fibroblasts with minor modifications, while others habitually grow as epithelial cells, forming sheets of connected cells either as membranes or more or less tubular structures. There may be exceptions to this behaviour, for fibroblasts occasionally join up or appear to join at the edges and form sheets,[98, 99] while occasionally epithelial cells display rather fibroblast-like growth, particularly when embedded in a semi-solid or solid medium.[184] As a rule, however, there can be distinguished a typically epithelial mode of growth which is clearly different from the growth of fibroblasts, and which is displayed by such tissues as the skin, glandular organs, and the mucous membrane of the alimentary canal. When epithelial tissues are cultured in simple fluid media, sheets of hyaline and more or less hexagonal cells may spread out on the medium-glass interface or

on the surface of the fluid, and the arrangement of cells is similar in appearance to that found in the so-called prickle-cell layer of the skin, except that the cells are all in one plane. The cells appear to be connected by fine protoplasmic bridges,[82] and as the culture grows the sheet of cells moves outwards as a whole. There is evidence too for the existence of a 'cement'

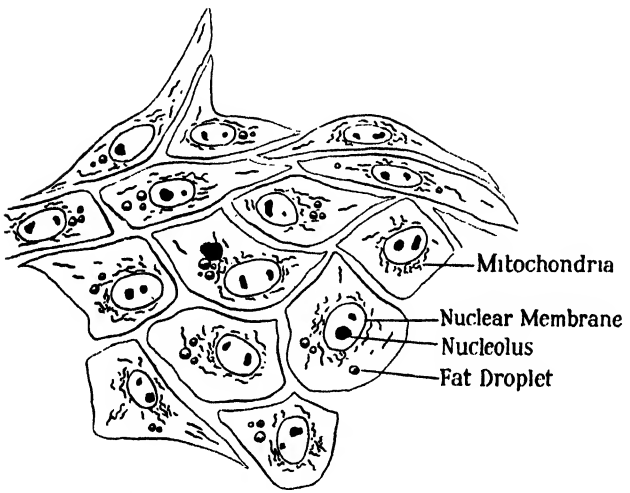

FIG. 4.—Diagrammatic representation of the cells at the periphery of culture of epithelium

substance lying between the cells, or of a condition favouring the deposition of silver in the spaces so formed. This silver impregnation at the margin of the cells should allow a distinction to be drawn between the epithelial cells and fibroblasts which may happen to resemble epithelium in their mode of growth. In most cases where the growth of fibroblasts has resembled that of epithelium there has been the possibility that endothelial cells were also present in the cultures.

(c) *Growth of Wandering Cells*

The wandering cells of the body form a third group of cells which can be distinguished by their structure and behaviour in vitro, but their rigid separation from other groups is beset with difficulties since, as will be discussed later, there is some evidence that so-called wandering cells may transform themselves into fibroblasts and vice versa. The appearance of wandering

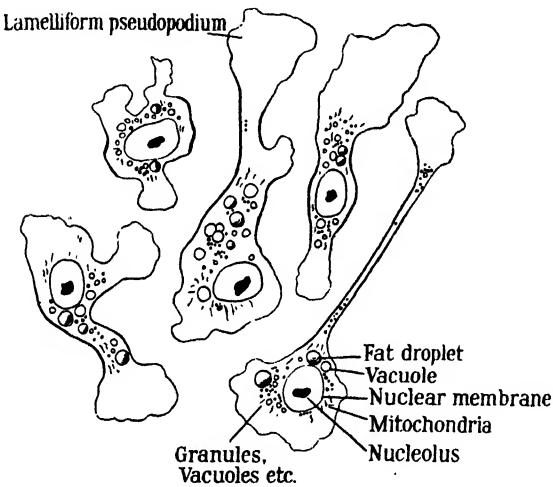

FIG. 5.—Diagrammatic representation of wandering cells in culture

cells depends even more than that of fibroblasts on the physical and chemical constitution of the medium in which they are living,[5] a fact which incidentally, from the histological and haematological standpoint, seems likely to have many and serious repercussions. Slight differences in form and staining properties would seem to fade into insignificance when the whole character of the cells may change with a slight difference in concentration of free amino-nitrogen in its vicinity. However, this subject will be again discussed on

page 94, and it is only necessary now to point out that as a general rule the wandering cells show sufficiently different characteristics from fibroblasts and from epithelial cells, to justify their separation into a group by themselves. In vitro, they behave far more independently of each other and grow scattered in the medium, while adjacent colonies tend not to fuse together, as often happens with fibroblast colonies, but to remain as separate entities.[44, 45] Under certain conditions, each cell shows undulating lamelliform pseudopodia [38] and its movement is rapid and erratic. There are other differences also, besides these which are more purely structural in character, but they will be discussed in connexion with differentiation and certain other aspects of growing cultures.

3. THE CELL COLONY AND THE MEDIUM

(*a*) *Non-growth-promoting Media*

These three main types of tissue, thus outlined, behave in their characteristic ways when placed in a medium composed not only of Tyrode solution but also partly of blood plasma. The plasma coagulates and forms a fibrin meshwork which offers further support for the growing cells, so that their activity is not then restricted to the glass or air surface but they can wander also into the substance of the coagulum. Owing to the proximity of the glass surface in a typical hanging-drop or flask culture the distribution of cells in the solid medium is rather uneven. With fibroblasts, for example, there is always an extensive outgrowth of cells between the plasma and the glass, a few irregular sprouts of growth from the sides of the culture, and then another layer of cells parallel to the glass surface but arising from the edge of the culture away from the glass. The cause of the formation of these distinct layers is not yet known, but light has recently been thrown on the question by the suggestion that a tissue

culture should not be regarded simply as a group of cells growing independently in a medium away from their natural position, but rather that it should be compared to a primitive organism, with a certain regulative capacity, that is to say, with a tendency to behave as a whole.[157] It might be natural to suppose that the cells of a culture grow outwards into the medium because, by so doing, they move to areas of higher oxygen tension, lower CO_2 tension, and a weaker concentration of metabolites. This in a solid medium would tend to give the culture a spherical form, which in practice it does assume when embedded in the medium far away from any surface, and it would seem possible to imagine other fairly simple forces, such as those caused by the proximity of the glass surface of the coverslip or flask, which might tend to flatten the sphere into the disk shape characteristic of well-grown cultures; but the necessity for an explanation of the curious distribution of the layers of cells away from the glass and the evidence obtained from a study of the repair of injured cultures indicate that other more complex forces enter into the situation, and that the culture must be regarded not as a collection of individual cells but rather as a colony which shows evidence of polarity, and of the existence of definite axes.[157] It is in some ways a disappointing conclusion, since it was at one time hoped that the methods of tissue culture would provide a means of studying cells carrying on their perfectly normal functions as almost independent units. But the idea of colony formation is borne out on other grounds also, for when small segments of the embryonic chick intestine are explanted into media of varying fluidity, it is found that they nearly always round up, surround themselves with epithelium and form little organisms. In fluid media these organisms tend to become hollow and cyst-like.[83]

When blood plasma is used in the medium it probably gives the cells an environment which is reasonably similar to that to which they are accustomed, and certainly offers them a much more uniform mechanical

support than they obtain in Tyrode solution, for it is certainly obvious that the behaviour of the cells in plasma coagula is far more constant than when they are provided only with a purely fluid medium. Helpful as it is in that way, however, it leads to obscurity in another, for plasma introduces into a comparatively simple system many and unknown complications. A Tyrode solution has a perfectly definite and repeatable constitution. Blood plasma is drawn from a living animal. It is a very delicately balanced system and undergoes changes even on standing; its properties vary with the age of the animal [36, 41] from which it is drawn, and its capacity for clotting to a jelly seems to be dependent on a variety of factors. For example, if it is allowed to clot alone, by warming to 37° C., it forms a loose coagulum from which fluid easily exudes under the influence of gravity; if it is clotted by the addition of traces of tissue extract the coagulum is much firmer, and fluid cannot be expressed easily for several days,[117] and then there is the possibility that it may arise at least in part from digestion occurring in the clots due to enzymes contained therein [57, 200] as well as by simple mechanical exudation of uncoagulable fluid. Plasma then, although of great convenience in tissue culture, is really rather a mixed blessing as regards many problems which might otherwise be attacked by in vitro methods. Its particular utility lies in its complete transparency, and in the fact that its constitution is apparently that of a meshwork of fibres, which forms in intimate contact with the tissue and along the threads or needles of which the cells can migrate outwards; that this is so is apparent from cinematographic records,[28] where cells can be observed to follow each other along definite paths into the medium. In the absence of a solid support, cells in tissue cultures tend to round up and float passively in the fluid, but when offered a suitable solid surface they will cling to it and spread themselves out along it. Not all solid surfaces are equally effective; glass, for example, should be free from all traces of grease; but such surfaces as

silk, spider's web, cotton-wool, &c., have been used with varying degrees of success.[92] If it is justifiable here to draw a parallel with somewhat similar phenomena in the animal body, it would seem that confusion may have arisen in the past by assigning definite shapes to various cell types. As a concrete example, is it not probable that the white cells of the blood and lymph are all spherical or nearly so, because they have no solid support upon which to cling, but that if they do by any chance stick to, or penetrate, the capillary wall they surely lose the spherical form and become amoeboid wandering cells? If this is correct it would be more reasonable to follow, and base distinctions upon, the history and behaviour of blood-cells rather than their shapes and staining reactions; for, after all, the latter may depend on the physical consistency of the protoplasm which may vary even from minute to minute according to the activity of the cell. Tissue culture, if it succeeds in no other direction, must certainly emphasize the dynamic nature of cells and tissues, and stress the importance of regarding cells as living and changing entities, which are largely at the mercy of their environment, and of each other.

If the tissue culture is to be regarded as an organized cell colony it would not be unlikely that there should be some limitations as to its size, and this is found to be so. Just as the fragment selected must not exceed certain rather small dimensions, so also is there a lower limit to its size. Single fibroblastic and epithelial cells do not seem to be able to survive alone.[11, 84] There are of course several possible explanations for this. In the first place, the mere process of isolation of the single cell may be sufficient to injure it irrevocably; secondly after the cell has been planted in the medium in which it is to grow, that medium may not be completely suitable. It might, for example, be too acid or too alkaline. Now if numerous cells were present they would probably be able to adapt the medium in their vicinity sufficiently in the right direction, possibly becoming slightly changed themselves. A single cell,

however, might not be able to produce sufficient acid or alkali to effect the necessary change. This perhaps is an extremely simple example of the interaction of cells with their environment, and it is further conceivable that there may be substances which are necessary for the well-being of the cell, and which diffuse slowly from one cell to another [87]; although it is a little difficult to see how the presence of another cell of the same type is going to benefit any individual cell in this manner unless it be in a very different state of functional activity. Cells of another type might on the contrary be necessary for proper functioning, and there is evidence that their presence may sometimes be extremely important although in another connexion. Fibroblasts periodically migrate away from the implant far out into the medium and, in such cases, although it is very difficult to be certain that they are really isolated and not connected by some very fine protoplasmic threads, they can often be seen to divide [200, 203]. This would seem to show that, given suitable surroundings, isolated cells may be capable of continued existence and even of division. There is no doubt, however, that the presence of other cells in the neighbourhood does appear materially to assist in the activities of any individual cell, although it is still uncertain whether the benefit is derived by diffusion through the medium, by contact between the cells, or by means of protoplasmic continuity. Moreover, when elongated threads of protoplasm are seen to be present, it is difficult to be sure whether or not the cells are really in direct protoplasmic connexion. One cell may merely be creeping over another. The evidence from microdissection suggests that the cells remain as isolated units, for injury to any given cell, although it may destroy it completely, is not transmitted to neighbouring cells.[46]

The essentially colonial type of behaviour and the dependence of one cell on another is also shown in a variety of other ways. When a culture of chick fibroblasts is made in a medium consisting only of plasma and physiological saline solution it visibly increases in

size. This increase is more apparent than real, being due mostly to the emigration of the cells from the centre so that in the periphery they may be only one layer thick, and the thickness of the central implant is much reduced. The actual number of cells is very little greater than at the time of explantation. In such a medium the change is one of reorganization from an isolated fragment of chick-heart tissue to what may perhaps be considered to some extent as a new organism. Cell division does occur but is not frequent and after a time ceases altogether. Cell emigration continues for a time after division has apparently stopped, but eventually after several days the culture ceases to spread further and the cells remain quiescent. If a part of the culture be cut away, the colony tends to repair the wound by increased cell activity, probably including a certain amount of cell division, in the immediate vicinity of the wound, and also the culture as a whole increases slightly in size.[71, 72, 93] The act of wounding the tissues seems to liberate substances which increase their growth activity. It may be said therefore that in this case there is nothing in the behaviour of the culture which suggests central organization because the whole process of repair could be explained simply on the grounds of the liberation of growth-producing substances, which are naturally in highest concentration near the wound and which also slowly diffuse into and stimulate the rest of the culture. Or again, when a culture is cut, there is always a retraction of the cells away from the wound, and this leads to local and temporary cell crowding which later has to be adjusted, and this might account for the repair. However, that neither of these is the complete explanation is indicated by the behaviour of injured cultures in a medium which is not only protective like plasma, but which actually stimulates growth, in other words in plasma and extract of embryonic tissues. If these cultures are cut with a knife the behaviour is very similar to that which has been described in plasma, but if sectors or parts of the culture are damaged or

killed by irradiation by ultraviolet light,[157] there is no retraction of the cell net and there is apparently no general stimulation of the growth of the colony, but the injured fragment tends to be restored to its original extent and, provided that the damaged sector does not occupy too large a proportion of the whole, the original circular form of the culture is restored. Here, there is no question of cell crowding consequent on the injury and subsequent attainment by the cells of their optimal density, and the absence of general stimulation of growth does not argue in favour of the liberation of growth-stimulating substances by the injured or dead cells. Rather does the evidence indicate that the culture has a tendency to form an organized unit, whose equilibrium shape is a more or less flattened disk of the type already described, and any tendency to depart from this condition is immediately counterbalanced by the increased activity of the cells in the regions where repair is necessary. It is as though the cells of a colony are constantly striving to push farther out into the medium, but beyond a certain limit the resisting forces become too great and they can penetrate no farther. Within the boundaries of the colony, however, the cells can move freely and so occupy the whole territory with ease, but beyond the boundaries they cannot readily penetrate. Apologies are offered for this somewhat anthropomorphic explanation, but in the absence of any concrete information as to the nature of the forces at work, such an explanation may give perhaps as clear a picture as any other.

Finally, in considering the culture as a whole, there are several other points which are of interest, in connexion with the ultimate size attained by the colony and the character of its growth. First, in media which are protective and not growth-promoting, as for example in plasma, the size of the original fragment determines the final size attained.[75] Where the medium is abundant, as in flask cultures, small fragments grow rapidly at first but soon reach their maximum size, while larger fragments do not make such a quick start but grow

for a longer time and finally show the same proportionate increase over their initial size as do the small fragments. In media which are growth-promoting, on the other hand, the final size attained by the colony, in flask cultures at any rate, is independent of the size of the original fragment.[73] After two or three days' growth, cultures from different sizes of explants show a remarkable uniformity.

In hanging-drop cultures [62] the behaviour is less regular; the smaller fragments are again more active at the start, but, contrary to what might perhaps have been expected, they show signs of degeneration earlier than do the larger fragments.

So far it has been tacitly assumed that fibroblast cultures of uniform size in plasma coagula all behave in the same way and only differ in behaviour in response to the presence or absence of growth-promoting substances. But there are other factors also which modify their behaviour, and much depends on the manner in which the cultures are made. As already pointed out, the plasma, when the culture is planted, coagulates and completely encloses the cells of the fragment of tissue in a solid jelly. By diluting the plasma with Tyrode solution it is possible to make clots of differing density, and by using different volumes, clots of varying depth. Both these variations bring about coincident changes in the character of growth obtained.[127, 197] Dilute and shallow clots lead to more extensive outwandering of cells, while concentrated and deep clots lead to compact cultures with very definite boundaries. Moreover, not only is the size of the colony affected but also the morphology of the cells. If the tissue is buried deeply in the coagulum or if the coagulum is dense, then fat makes its appearance in the cells. The origin of the fat is still a debatable question; it could either come from the medium directly, or by transformation from proteins or carbohydrates in the medium, or it could arise by the unmasking of the fat in the cells themselves. And here again are at least two possibilities. Perhaps the cell fat is merely rendered visible by a

physical change in its distribution, or perhaps it is manufactured by transformation from other cell constituents. At present it is not known. An interesting observation has recently been made in connexion with the appearance of fat in the cells.[118] It has already been mentioned that if a pure plasma clot is placed in a vertical position so that liquid can drain away from it, quite a considerable quantity of fluid is expressed. Cultures placed in such vertical coagula grow quite normally and the cells, particularly those in the lower sector, remain hyaline and free from fat, whereas if the clot remains horizontal, so that the fluid cannot escape, the cultures grow as usual and the cells all show more or less fat. Finally, in cultures in plasma and embryo extract, a gradient of increasing fat deposition in the cells towards the periphery [62] is nearly always noticeable, particularly in hanging-drop cultures or in shallow clots. This does not occur so readily in media containing plasma only, and since the cells near the periphery have a high rate of division in cultures containing extract, the deposition of fat in this case looks like a sign of good feeding (!) rather than a degenerative process as it may be in other cases, as for example after irradiation by ultra-violet light when the cells round up and become laden with fat.[158]

So far, practically all that has been said has referred to the general behaviour of tissues in media outside the organism, and before proceeding further some of the more essential conditions for the survival of tissues in vitro may, at the risk of repetition, be enumerated. With regard to the tissue itself, almost any type can with suitable handling be made to survive for an appreciable time when removed from the body; even nervous tissue which is notoriously sensitive, will under suitable conditions not only survive but regenerate fresh axons and processes.[103, 114] For all tissues, size is an important consideration; the fragment must neither be too great to inhibit oxygen from reaching the centre, nor too small to be able to equilibrate itself with the medium, though on this last point there is a

difference of opinion, and further work is necessary. With regard to the medium the salt and ionic concentration must be within physiological limits; in this connexion it is interesting to note that tissues can show remarkable powers of adaptation for short periods, but prolonged sojourn in a medium which is far removed from the normal leads to decreased activity and eventually to death. For instance heart tissues were not found to be killed immediately by NaCl concentrations as widely different as 0·2 per cent and 1·2 per cent, although in the former the cells became highly vacuolated.[196] For prolonged existence any concentration far removed from 0·8 per cent sooner or later proves fatal.[63] Sodium, potassium, and calcium ions must, as might be expected from analogy with other physiological work, all be present in the medium in correctly balanced proportions. Again, although the tissues which have been investigated will stand a great range of acidity or alkalinity (*p*H changes from 5 to 9)[139] for short periods, yet prolonged survival will only take place when the medium is nearly neutral (*p*H 7·4 approximately).[92] The medium in the immediate vicinity of the tissues often shows a different reaction from the rest of the medium and is generally more acid. For tissues from warm-blooded animals the temperature should be maintained as near that of the animal as possible. Cell activity for warm-blooded animals generally commences about 20° C. and rises to a maximum at about 40° C. or perhaps a little higher. Cooling the tissues to 0° C. for several days has no permanently injurious effect,[89, 126] although of course, at the time, all visible signs of activity disappear, and cultures kept for long periods in the cold chamber often become vacuolated and only recover slowly.

(*b*) *Growth-promoting Media*

Granted then that the normal conditions outlined above are observed, tissues will survive for as long a time as their reserves last. In plasma, tissues may be kept alive indefinitely provided that the coagulum is

either frequently renewed or that it is washed and fed repeatedly with either serum or, better, plasma that has been treated with the anticoagulant heparin.[95] Plasma, then, and probably serum, can give to cells everything which they require to maintain life. As will be seen later, the cells under these conditions not only survive but they undergo differentiation, and to some extent carry on their normal functions. The process of differentiation will be discussed in more detail but the interesting point now is that under conditions which allow of differentiation, the cells do not multiply very rapidly, if at all. The growth rate in plasma alone quickly falls and the cells cease to divide. Thus although growth and differentiation are processes which normally go hand in hand, and in vitro can frequently be found to be so doing, they are in reality essentially separate mechanisms, and conditions suitable for one are not necessarily those that are suitable to the other. In fact, when growth by cell division is occurring in vitro most actively, that is, under the influence of embryo extract, there is very little sign of differentiation and the phenomenon goes by the name of unorganized growth. Moreover, cells have been kept growing in this condition for a great many years [65] and, apart from the possibility of accidental infection, &c., there is no apparent reason why they should ever stop. Unorganized growth therefore opens up many problems and for a long time in the early history of tissue culture it occupied the centre of the stage.

The key substance which allows this indefinite growth is of course the extract of embryonic tissues in physiological saline solution which has already been frequently referred to as embryo extract. The addition of traces of this extract to cultures growing in plasma or even Tyrode solutions produces a great increase in the growth rate, probably also in the general activity of the cells, and in mixtures of plasma and extract this high growth rate can, by suitable technique, be indefinitely maintained. How the extract produces these effects is still wrapped in the mists of obscurity, which, although

they frequently appear to be lifting, come down again as thick as ever. Growth under the influence of embryo extract is mainly by mitotic cell division, and since cultures growing in such a manner are exceptionally

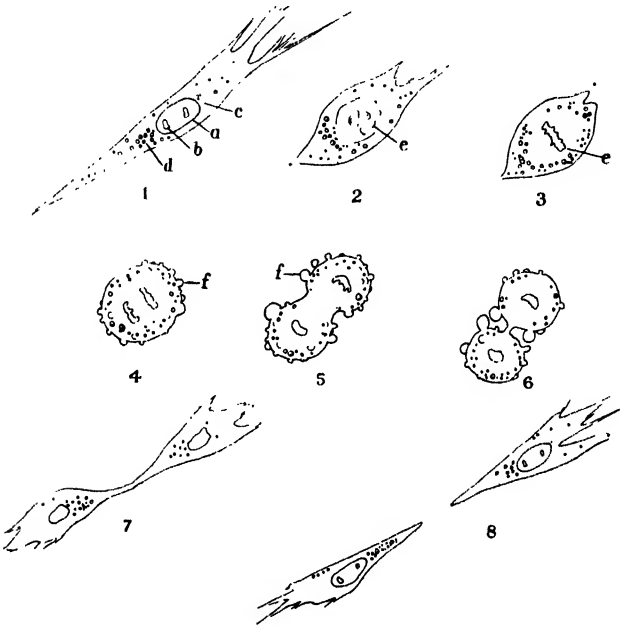

FIG. 6.—Diagram illustrating the chief phases of mitosis as seen in fibroblasts growing in vitro

1, resting cell; **2**, prophase; **3**, metaphase; **4**, early anaphase; **5**, late anaphase; **6**, early telophase; **7**, late telophase; **8**, daughter cells. **a**, nuclear membrane; **b**, nucleolus; **c**, centrosphere; **d**, fat; **e**, chromosomes; **f**, bubbles

favourable for the study of mitosis, it may be pardonable to digress at this point and discuss the influence of tissue-culture methods on our knowledge of the process of cell division.

Apart from such cells as the ova of certain marine or cold-blooded animals, very few, if any, animal cells

can be observed in a state of division in a manner which is anything like so clear as that afforded by tissue-culture methods. Direct observation [140, 179] and suitably "speeded-up" cinematograph films [28] of cells in culture have given a very complete visual picture of the process. The cell that is about to divide, a typical fibroblast for example, is first generally conspicuous among its neighbours by its larger size, and then because it starts to retract all its processes. Occasionally small droplets of protoplasm are left behind as the processes withdraw, but generally the whole cell becomes more globular. This must indicate an increased surface tension or gelation of the protoplasm from a central point. Concurrent observations on stained specimens show that this rounding-up stage is coincident with prophase of the nucleus in which stage the chromosomes are making their appearance. The actual nucleus and chromosomes are very difficult to observe in the living cell at this time. The fixed cell apparently stains rather more deeply during mitosis than at other times, but this may very well be, and indeed probably is, an artifact since, owing to the assumption of a spherical form, the optical depth of the cell is greatly increased. Similarly, staining by cobaltinitrite for potassium gives an apparent increase of this ion in the cytoplasm at mitosis but this increase is again probably not a real one. By the time that the rounding up of the cell is complete the cell is in metaphase and the chromosomes may be distinctly seen as highly refractive bodies lying at right angles to what was originally the long axis of the cell. They are arranged at the equator of a double cone of clear gelatinous protoplasm known as the mitotic spindle. Very shortly afterwards anaphase commences and the two chromosome groups can be seen to separate and move apart towards the two poles, but before they have quite reached their destination changes take place in the equatorial region of the cytoplasm. A constriction forms, which quickly deepens and as it deepens the whole surface of the cell is affected by change. Small

bleb-like pseudopodia are thrust out and withdrawn, from all over the surface. Compared to the normal rate of formation of pseudopodia, this process is extremely rapid, and the speeded-up cinema film provides a very curious spectacle. As the nuclei re-form in telophase and the two daughter cells separate, they are, at the last, held together by a narrow isthmus of protoplasm and on either side of this the bubbling of the cytoplasm is generally most intense and in the film gives the appearance of the cells trying to force each other away as though engaged in a boxing match. Of course it need hardly be said that this speeding up gives a very exaggerated picture, but the local changes in surface tension or whatever are the causes for the formation of the blebs are certainly remarkable phenomena. Moreover, it is of particular interest here since it was first noticed in connexion with cells in tissue culture. All the visible changes in mitosis are normally over in about thirty minutes, but the time is, of course, affected by temperature,[121] and similar temperature effects are noticed on mitosis as on the activity of the culture as a whole. The effects of raised temperature are at first reversible but become irreversible if continued for too long, or if the temperature is too high. Cells in mitosis cannot safely be heated above 50° C. During the various phases the cells are not equally sensitive to temperature and the process is most easily checked in metaphase, so that the cells do not enter anaphase. This may be connected with the consistency of the spindle, which has been shown to be changed by heat with the formation of a gelatinous network.[137] Abnormal mitoses are not uncommon in tissue cultures. In particular, tripolar mitoses are not infrequent,[49, 120] a phenomenon which would seem to rule out any magnetic theory of mitosis, although it has been claimed that the movements of the chromosomes are susceptible to electric fields.[21] Often the cytoplasmic behaviour does not follow that of the nucleus. Small portions of cytoplasm may be extruded during division and both daughter nuclei remain behind in one cell, leading thus

to the formation of bi-nucleate cells.[180] In a similar way giant cells with many nuclei may be formed, namely by repeated nuclear divisions which are not followed by cytoplasmic division. It has been suggested[143] that such nuclear division may be amitotic, each nucleus simply splitting directly into two parts as the result of a deep constriction in the equatorial region. Signs of such a process have been frequently observed in fixed tissue-culture cells, but generally in degenerate cultures. In the living state the phenomenon has only rarely been observed.[143] In any case a cell which has once divided in this manner probably never divides again by the usual method of mitosis. Some observers have formed the opinion that direct fusion of normal cells rather than, or perhaps as well as, the failure of division is a regular method by which at least one type of giant cell makes its appearance, and the cells so formed are characterized by the regularity in size of their nuclei.[141] The formation of giant cells seems to occur very readily among certain cell types, particularly wandering cells, when the cells are treated with foreign particles such as lycopodium powder, certain bacteria, &c.,[141, 125] and also in response sometimes to lack of oxygen, increased CO_2, or some other factor connected with the depth at which the cells lie in the coagulum.[12] By culturing tissues at different depths from the surface of the coagulum giant cells are formed more frequently among the cells far removed from the surface. Although, with the exception of giant cell formation, the preceding descriptions of cell division, &c., have been made with particular reference to fibroblasts, they apply almost equally well to cells of other types. In the case of wandering cells the bubbling phenomenon in mitosis is even more rapid and violent than in fibroblast. Possibly it is due to small local changes which lower the surface tension very markedly in their immediate vicinity and then are quickly reversed. When the bubbling ceases the lowering becomes general and the more or less globular daughter cells suddenly flatten out once again on the supporting surface, send out

pseudopodia into the medium, and immediately begin to draw apart. After a short time the one daughter cell, which is then necessarily migrating more or less inwards towards the centre of the explant, changes its direction of motion and commences to wander outwards once more. In this way the two daughter cells are seldom to be found immediately adjacent to each other even a few hours after division, although they are seldom far apart.

Such then is a brief account of the chief phenomena observed in the process of mitosis by tissue-culture methods. Has any light been shed on the actual nature of the process by experiments on cells in vitro? Experiments with microdissection needles on resting cells [46] have shown that at least in the case of fibroblasts there is a distinct mechanical membrane to both the cytoplasm and to the nucleus. In the wandering cells, the cell membrane is much less rigid, and moreover these cells attach themselves to surrounding objects practically by their whole surface, while fibroblasts are attached only at the extremities and are apparently in a state of considerable tension, so that injury sooner or later produces a sudden and quick retraction of the cell processes. In this connexion it may be mentioned that the cytoplasm of the wandering cells is more fluid than that of fibroblasts. The membrane of a fibroblast which is undergoing mitosis is similar in character to that of the 'resting' wandering cell, in other words it is much less rigid and shows rapid outpocketings and indrawings. A similar phenomenon occurs in degenerating cells. In fact, superficially, perhaps actually, there is a great similarity between cells which are rounding up to divide and those which are rounding up prior to degeneration. In both types the cell surface appears to become unstable and eventually shows the bubbling phenomenon. In certain conditions of culture, as for example during prolonged culture in plasma alone, cells will often round off and stay in that condition for an hour or more, and then continue a normal division to produce two healthy-

looking daughter cells. The normal time during which a dividing cell is spherical is only a few minutes,[203] and the long delay in this phase in cultures which are rather exhausted or not growing actively is interesting. In the stage when the cell is spherical it seems that a slight turning of the scale in one direction or the other is sufficient to send the cell either towards normal division and a new lease of life on the one hand, or to degeneration and death on the other.[156]

It is unfortunate that the normal chicken fibroblast shows a very poorly developed spindle during mitosis, but it is generally evident that after the nuclear membrane disappears a mitotic spindle does form as a distinct, homogeneous, hyaline, and gelatinous body. In the living cell there are normally no traces of any fibres in this spindle, that is to say, there are none of the fibres to which the chromosomes are apparently attached when seen in fixed preparations, but they may be caused to appear when the cells are irrigated by an acid medium.[136] At first the process is reversible so that on restoration to a normal medium the fibres disappear. With stronger acid the cell is damaged and the fibres persist. A variety of other reagents will cause spindle fibres to appear in a similar manner, such as salts of heavy metals, and alcohol.[138] The spindle may be caused to disappear on treatment with hypotonic solutions, and the chromosomes become scattered at random, but they can be collected again, so that normal mitosis can be resumed when the correct osmotic pressure is restored.[138] The cell that is dividing or about to divide is more sensitive than the resting cell to changes in its physical environment. Thus researches on the effect of lowered temperature [177] and of X-rays [148] and radium [29] on the occurrence of cell division show that the cells are not equally sensitive at all times to these reagents, and the fact that, with certain treatment, cells already in mitosis can complete their division while hardly any more cells enter into this state suggests that there is a specially sensitive period just prior to division. This inhibition too may be only

temporary, for if the culture after treatment with cold, for example, is restored to its normal temperature there follows a period when divisions are excessively abundant. In fact, the deficiency in divisions caused by the stay in the cold is almost exactly compensated for by the excessive divisions which follow when the normal surroundings are restored. This, of course, indicates that in the period between mitoses the cell is not affected until just when the process is about to occur, so that all those cells whose division has been temporarily inhibited divide at the same time as those which would, and do, divide in the ordinary course of events. Exactly the same compensatory phase follows a period in which mitoses have been inhibited by X-rays or radium.

Although tissue culture has contributed but little so far to the available information concerning the actual mechanism of mitosis, when suitable methods of attack are devised it should prove to be very valuable, since it offers cells in mitosis in a very accessible form. Meanwhile, the formation of the spindle, the movement and final separation of the chromosomes, and the division of the cytoplasm are still processes in which there is almost everything to explain. On the other hand, a considerable body of information now exists concerning the distribution of mitoses in space and time during the growth of a culture.

The standard method [64] by which the size of a colony is estimated, and thereby some measure of its growth obtained, is by recording its surface area at stated intervals. The method is convenient, for the culture is placed in some form of projection apparatus and its area measured by a planimeter or by means of squared paper, and the process can be repeated as often as necessary. On the other hand it is a method which is open to many and grievous objections. Growth of the culture is three-dimensional, and not uniformly so. The proportion of growth in the three directions is not only unequal, but probably there is no constant relation between the amount of growth in the third direction

and its extent in the other two. But even supposing that there were such a direct and determinable relation, measurements of area only might still give very false ideas of the exact state of affairs in the culture. The migration of the cells has to be taken into account, and, unless division of cells is occurring rapidly, it may be found that after several sub-cultures the tissue no longer maintains its size, because in the past the apparent increase in size of the culture has depended for the most part on the spreading out of the cells. Consequently for reliable data on growth by measurement of area only, the experiment should be long continued and account taken of the possibility of prolonged sub-cultivation without loss of size of the culture. Another factor of importance is cell disintegration. It has been shown that there is at any rate a superficial resemblance and connexion between cell disintegration and cell division, and it is possible that a high division rate may be closely connected with a high rate of disintegration, a process which might well be autocatalytic since the processes of cell breakdown are known to provide conditions suitable for building up new cells. Extracts of autolysed tissues [10, 61] are said to be strong growth-promoters. Moreover one particular case [94] has been worked out which is very illuminating, although perhaps not as well controlled as is desirable, since two types of tissue which are manifestly dissimilar were compared. The growth rate of a colony of chick fibroblasts was compared with that of a mouse carcinoma, by the method of area measurement, and the former was found to have a considerably higher growth rate. The cultures were then killed and sectioned and the number of cell divisions counted throughout. The results were interesting from two points of view. First, the mouse carcinoma culture showed far more divisions than that of the normal fibroblasts, although its apparent growth rate was less, and secondly, light was thrown on the spatial distribution of dividing cells throughout the cultures. Presumably, although it was not definitely established, the high rate of cell division in the carcinoma

tissue was offset by an equally high rate of cell disintegration, so in studying growth rates of tissues it is necessary to take all these factors into account.

In a normal chick-heart fibroblast culture growing in embryo extract the region of most intense cell proliferation is just removed from the periphery of the culture. The cells which are farthest out in the medium are often large and irregular in outline. Divisions frequently occur in them too, but it is the cells which are not quite so isolated which seem to show the highest rate of division. In fact, there is a distinct optimum density of cell population for division.[198] Cells towards the centre of the culture again show divisions but not nearly so abundantly; and the cells in the centre of the original tissue often show no divisions at all.[94] These facts all illustrate the difficulty of interpreting the measurements of the area of a culture, as a measure of actual growth by increase in cell number. Several workers by making assumptions as to the rate at which cell division is occurring, have elaborated formulae connecting the area of the culture and its rate of growth.[1, 75, 147] These are always more or less unsatisfactory as the area depends on at least two very variable factors. The rate of migration of the cells away from the centre, even in embryo extract, where cell divisions are numerous, may account for as much as sixty per cent of the total increase in cells in the peripheral zone, and the rate of cell division itself is constant neither in time nor space.

Some light has been thrown on the processes involved in the growth of a culture by obtaining continuous photographic records of the behaviour of the cells in the outer part of the growth zone.[198, 199] The cells in this region were chosen because they alone are sufficiently separated and distinct to provide clear photographs. It is fortunate that they also happen to be the cells which are in closest contact with the medium and probably less under the influence of changes in the central implant, and also that they are those which in suitable media show a high growth rate. It is possible to count

the cells in photographs of this region so that the growth rate can be numerically expressed as the percentage of the cells present which divide per hour. Since the photographic field may conveniently hold up to three or four hundred cells, and may be occasionally moved so as always to include the periphery, the method gives some idea of the actual manner in which a cell colony is growing, and the relative parts played in the process by cell division, migration, disintegration, and increase in cell size. Even so there are, unfortunately, of necessity several inaccuracies. The number of cells is really too small; only part of the periphery of the culture can be included, and there is always, from culture to culture, a certain amount of unaccountable variation. However the method is definitely useful in elucidating the behaviour of cultures under different conditions. A culture of chick-heart fibroblasts removed from a nine- or ten-day-old embryo and explanted in a fowl plasma coagulum which is then irrigated with Tyrode solution shows in its periphery, as mentioned previously, a progressively decreasing rate of growth. From the time when sufficient cells have emerged into the medium to make it possible to take photographs, after say twenty hours, the growth rate is of the order of four per cent and falls rapidly till, at the end of fifty or sixty hours, mitotic division has practically ceased. If the culture be treated with embryo extract instead of Tyrode solution the growth rate is considerably greater, starting even as high as nine or ten per cent and not being reduced to zero for five or six days. In fact, if the embryo extract is renewed every two days, which is the usual practice in the maintenance of tissue cultures in flasks, the rate of growth fluctuates around three per cent for as long as it has been possible to obtain photographs, a period of about ten days. After this time, owing to slight precipitations from the proteins of the extract, the coagulum becomes somewhat opaque, and the photographs correspondingly obscure. It is of interest to compare these figures with those obtained by examination of fixed and stained preparations both

of the chick heart at various stages of its development in ovo, and of sections of cultures in plasma and extract,[169] which take into account the behaviour of cells in the central implant. In these cases the actual figures were obtained as the number of cell divisions visible per thousand cells, but allowing for the fact that a cell division lasts for about thirty minutes the figures can be compared with the growth rates given above. When these necessary corrections are made for the entire chick heart, it is found that on the second day of incubation the growth rate is rather over four per cent and falls steadily, with the exception of a possible slight rise about the tenth day, to negligible quantities soon after hatching. The growth rate in the periphery of a tissue culture is therefore considerably higher than any normal growth rate for heart tissue; for the ten-day chick heart the value is about two per cent so that even the figure three per cent obtained during continued cultivation is higher than normal. Taking the culture as a whole, however, the division rate in vitro is not as high as in the normal heart of the very young embryo for the cells in the centre do not maintain the high rate set by the cells on the periphery. But whereas the growth rate of the embryo steadily falls off, in vitro it may be maintained constant at about 0·8 per cent for very long periods, so that if a culture is made from a fifteen-day chick heart, by which time the growth has fallen to about 0·8 per cent, after a few days the culture is growing at a much faster rate than the intact heart, in which divisions may have almost ceased. It must, however, be remembered that in the intact heart there are several types of tissue present, all interacting with one another, and it is uncertain whether all cells are represented in vitro, and if not, which are the ones with which comparison should be made. The high rates of growth often found in the very early stages of a culture may in part be due to the process of explantation, in which some cells are certainly damaged and probably their disintegration products stimulate the remainder. Another factor

which may be involved is the possible delay in division caused by the handling of the tissues which leads to an outburst of divisions when recovery takes place, just as occurs after cooling or treatment by X-rays.

There is at present no information as to what happens to the growth rate of a culture after ten days in the same plasma coagulum, when only the embryo extract of the fluid phase is renewed. Generally at about that time measurements of area show a sudden cessation of the hitherto regular increase, so that possibly the growth rate declines rapidly too. The failure of a colony to increase further may in some way be connected with an optimum or equilibrium size, in which cells have attained the necessary spacial distribution, so that they are neither too crowded nor too isolated, and therefore have ceased to migrate outwards any further, except just to compensate for the new cells which are constantly being produced. Or on the other hand the cessation of growth may be genuine and due to the exhaustion of some factor brought in by the plasma. Much could be added to the present knowledge of the behaviour of tissue cultures as a whole by a satisfactory elucidation of this point.

Another important factor which is brought out by this analysis of growth rates and which has also been found experimentally by direct observation of single cells [168] is that any conception of anything more than a crude average intermitotic period, or the use of the intermitotic period of a few cells as a measure of growth rate, is definitely erroneous. The growth rate and intermitotic period are things which rapidly change according to the age of the culture, &c., and as instantaneous measures they mean very little. For example, cells have been observed to divide again after as short a time as seven or eight hours after the previous division, others have not divided over a period of twenty-four hours, and possibly some only divide very rarely, if at all. The criticism might be levelled that most of the above figures refer to cultures from fresh tissues which therefore probably contain cells of many kinds and

cannot be regarded as pure cultures. However, direct measurements of the intermitotic periods of cells in cultures from a strain of fibroblasts which has been cultured for over twenty years,[168] also emphasize the extreme variability from cell to cell, and that any uniformity in this respect in the composition of the cultures is not to be obtained even by prolonged cultivation. Moreover, care must be exercised in making measurements of growth by counting the numbers of mitoses in fixed preparations, unless these are all fixed at exactly the same period in the life of the culture. It so happens with hanging-drop cultures in plasma and extract that although the rate of cell division constantly declines, yet the number of cells in the periphery of the culture increases owing to the results of division and migration, and at such a rate that in this medium the total number of mitoses stays remarkably constant from the twentieth to the fortieth hours after explantation. But this is a special case and is probably untrue for most other media. If a tissue culture is grown in plasma only until the cells have ceased to divide, and it is then treated with embryo extract, the division of cells recommences, but not immediately. There is a latent period of about ten to twelve hours after the addition of the extract before the peripheral cells show the increase in division rate. About twenty-four hours after the addition of the extract the growth rate reaches a maximum and then falls away again to zero after a further twenty-four hours. The time of occurrence and value of this maximum vary with the strength of the extract and also with the type of tissue. Gradually increasing movements can be observed in the peripheral cells some hours after the addition of the extract, but divisions do not occur for at least ten hours, and moreover this latent period does not appear to be correlated with the duration of the ' starvation ' period before the addition of the extract. The cells living in plasma alone are relatively inactive and closely packed together, but life may be maintained for a very long time, particularly

when the cultures are periodically washed and fed with fresh plasma which has been treated with heparin to prevent coagulation. The conclusion would therefore seem justifiable that cells in plasma are not cells which are really under starvation conditions. On the other hand they are in a relatively quiescent state. On the addition of embryo extract the cells soon become much more active and migrate freely into the medium, and eventually start to divide. These two facts suggest that activity and division might depend on something more than the mere nutrition of the cells. It would appear that embryo extract provides something that the cells normally do not possess, or that they quickly lose under conditions of culture. This substance or combination of substances acts as a general stimulant causing both increased movement and division, but it cannot exert its effect immediately. Is this delay due to its slow penetration into the cells, or does it set in action some synthetic process within the cells which eventually culminates in increased activity? Moreover, the substance can be extracted in greater quantity or in a more active form from embryonic tissues than from most tissues of the adult animal. Presumably this is correlated with the declining growth rate which occurs in the development of all animals, and is reflected in tissue culture by the decreasing residual growth energy of tissues with increasing age. Broadly speaking, the younger the tissue the more readily does it grow in vitro, although there may be minor discrepancies owing to the different times at which different parts of the body are developing and differentiating most rapidly.[10] Adult tissues which have normally ceased to grow or nearly so, are very inactive when cultured in vitro, but the addition of an extract of embryo tissue sooner or later once again stirs them into activity. This too suggests something out of the ordinary run of nutritional substances, and that the needs of tissues fall into two categories, those for maintenance and those for growth. The hypothetical substance for growth might be a food substance, but is

probably more likely to be something which alters the metabolism of the cell in some way. It is not species specific; that is to say, embryo extract derived from chick tissue will equally well stimulate the growth of rat or human tissue.[42, 85]

4. METABOLISM OF GROWING CELLS

(*a*) *General Characteristics*

The metabolism of cells growing in vitro has been very little investigated, chiefly owing to the technical difficulties involved. Successful growth is only possible when the tissue has something upon which to cling, and since the substance used is generally plasma, from which it is almost impossible to effect a complete separation of the tissue when required, measurements of the weight of the tissues are extremely difficult, and this renders any tissue analyses correspondingly vague. For several investigations more or less indirect methods have been adopted.[128, 144, 159]

All living cells require energy for their very existence, and tissues in vitro are no exception. The energy is obtained by the cells of higher animals for the most part by the oxidation of carbohydrates, sugars, &c., so that, ordinarily, tissues require oxygen and a carbohydrate for maintenance, but there are certain types which can for a limited time obtain energy from an anaerobic conversion of sugar into some substance like lactic acid (cf. yeasts and the formation of alcohol),[190] and if provided with sugar such tissues can therefore exist with minimal supplies of oxygen. Often the formation of a substance like lactic acid can be only temporary since if it accumulated it would damage the tissues. Tissues which in air break down sugars directly to CO_2 and water are said to obtain their energy by respiration; those which form lactic acid or some similar body in air are said to show aerobic glycolysis, and those which form lactic acid under anaerobic conditions show anaerobic glycolysis. Tissues from very young embryos seem to have some capacity for living

completely anaerobically.[192] For example it has been found that the chick heart if taken from an embryo of less than five days' incubation and cultured in vitro [24] will grow under completely anaerobic conditions, or at least will display movement of the cells out into the medium. Certain cancerous cells behave in a similar manner,[191] but for most tissues oxygen is required. The survival of those tissues which have any capacity for anaerobic existence is naturally very much assisted by the addition of glucose to the medium, for with a liberal supply of this substance energy can be obtained without the use of oxygen, but the process is not so economical as the direct oxidation of sugar, and therefore demands a much greater supply of the raw material. In the absence of sugar, some tissues, as for example the embryonic kidney, have a limited capacity for burning nitrogenous bodies,[107, 193] at least ammonia is found to be formed by such tissues when sugar is only present in minimal quantities in the medium, and so long as the tissues show visible signs of activity by increasing in area. The formation of ammonia ceases when the tissues become inactive, or when sugar is added to the medium. Embryonic tissues thus show a preference for carbohydrate as a fuel, but in its absence can make use of other sources of energy of which there is definite evidence for the utilization of nitrogenous bodies, possibly proteins.

(*b*) *The Source of Nitrogen*

This then opens up the question of the normal source of nitrogen for cells living in vitro. When active growth is occurring considerable quantities of new protoplasm are being formed, of which protein is presumably one of the major constituents. In the body it is believed that the proteins are obtained by synthesis from amino-acids. This assumption depends chiefly on the fact that in the alimentary canal the proteins are broken down to their constituent amino-acids by means of the enzymes found in the digestive juices, and moreover amino-acids are found in the blood. It

would be natural to expect that tissues in vitro would also use amino-acids as their raw material, but the evidence in favour of this, and indeed concerning the whole nitrogen metabolism of the isolated tissues, is very unsatisfactory. One important point has to be kept in mind in nutritional experiments of this sort. In the body there is a very large blood-stream, with which the lymph and tissue fluid are potentially in equilibrium, and upon which the tissues can constantly draw for fresh supplies. It follows then that extremely minute concentrations of certain substances may be sufficient to satisfy the needs of the cells because the concentration though minute is maintained. To draw a parallel, vanadium is present in the sea in infinitesimal amounts, and yet certain tunicates [106] can extract sufficient of it to make use of it as a respiratory (?) pigment. The substances which are utilized in this way might very well be toxic if supplied in greater concentrations. Perfusion methods [23, 52] have been described for maintaining cells in vitro in a state of prolonged activity outside the body, but for technical reasons of one sort or another they have not yet, except in one or two instances, been used to any great advantage, so that definite information on the effects of small quantities of nutritive substances on the behaviour of the cells is at present sadly lacking. A point which is worth consideration in this connexion is that during prolonged cultivation in flasks, when a fluid phase of the medium above a solid coagulum is renewed at frequent intervals, the growth, judged by the numbers of mitoses, is more or less rhythmic. There seems to be evidence that at each change of medium the growth rate is low, and may even decline at first, and it then rises to a maximum about twenty-four hours after the change. Admittedly the evidence for this is still somewhat meagre, but it is at any rate suggestive. If it is true, it indicates that the media supplied to the tissues are not really exactly those that the cells require, and that at each renewal the cells have to adjust themselves to the new conditions, or possibly that some substance

supplied is, after each change, present in too high a concentration for the greatest cell activity, which can therefore only be displayed when the concentration has been somewhat reduced.

To return again to amino-acids and their effects on fibroblasts in vitro, the earlier experiments failed,[27] since the concentrations used were manifestly too high, and the concurrent hydrogen ion concentration was insufficiently controlled. Later it has been stated that amino-acids produce a slight and temporary stimulating effect,[66] but do not produce any increase in cell mass, nor maintain life in the tissues for longer than simple salt solutions. This temporary stimulation may be important. Indeed the addition of glycine [9] to cultures being fed with peptic digests of protein exerted a very markedly beneficial effect. It is known too that dialysates of embryo extract produce a similar stimulating effect,[6] and also that numerous cell divisions can be observed in cultures treated with them.[202] The activity of such dialysates has been attributed to their content of amino-acids.[6] Obviously then these substances cannot be summarily dismissed from the picture, although perhaps they are not the chief factors in cellular nutrition. It would indeed be odd if the main nitrogenous food substances of the organism were completely without effect on the isolated cells of that organism. Rather is it necessary to consider the negative evidence with extreme care, before thus ruling them out altogether. The same applies, only with less force, to the negative effects obtained on adding proteins to the medium, since the animal body always appears to have to break down these bodies before it can utilize them. In cultures there is very little evidence that proteins of any sort added to the medium are in the least beneficial to the cells,[7, 134] with the possible exception of egg white; this substance, when suitably diluted, is under certain conditions a superior medium to simple Tyrode solution. However, this is by no means a pure protein, and there is reason to suspect that it may owe its activity to substances other than the albumen.

It is unlikely, and there is as yet no evidence in favour of the supposition, that cells utilize nitrogenous bodies of simpler character than amino-acids, e.g. such substances as urea, nitrates, &c.; neither do proteins form the immediate nitrogen supply, so that presumably the substances intermediate between proteins and amino-acids are the ones that are important, and there can be no doubt now that in tissue cultures these substances can be extremely important. When various proteins [3, 9, 35] are digested by means of pepsin, the digest adjusted to suitable salt concentration and acidity, and fed to fresh explants of fibroblasts growing in plasma coagula, there is an enhancement of the growth of those cultures, which might lead to the supposition that intermediate protein-breakdown products may form the chief essentials for life. In general this may be true, but there are certain limitations which indicate that not every intermediate product is of equal importance. In the first place for maximum results the ratio of the amino-nitrogen to total nitrogen in the digest must not be too high, or in other words the digestion must not have proceeded too far.[10] It is definitely among the higher split products that the effective substances must be located, and tryptic digests, or those which have a high proportion of peptones or free amino-acids, are relatively inert. It is the proteose fraction which contains the substances which appear to provide the cells with their chief requirements, but all proteoses are not equally effective. Although digests of several proteins have been tested, among which is included that from crystalline, and therefore fairly pure, albumen,[9] yet those from commercial blood fibrin generally produce the most striking results, and indeed Witte's peptone, which is made from this source, when suitably applied to the cultures, forms quite a satisfactory medium. Perhaps there is some extraneous body associated with blood fibrin which increases its activity. In contrast to the behaviour of tissues in plasma and embryo extract, in which they grow and remain alive indefinitely, in proteose solutions their period of

survival is limited. Although at first the cultures live actively, often very actively, in such media, sooner or later the cells become fatty and eventually degenerate. Cultures too which have been maintained in vitro for

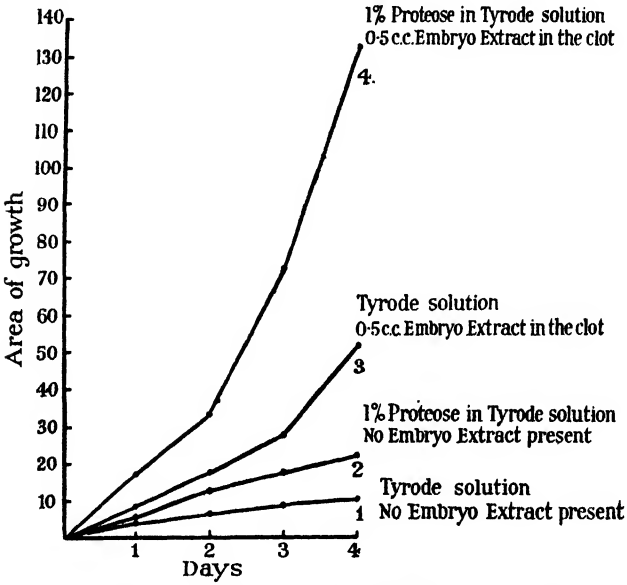

FIG. 7.—Diagram showing the area attained by cultures of chick heart fibroblasts in media containing **1**, plasma and Tyrode solution; **2**, plasma and proteoses; **3**, plasma and embryo extract; and **4**, plasma, embryo extract, and proteoses. The areas of growth are in arbitrary units, being the number of squares occupied by the image of the culture in the camera lucida

any length of time cease to benefit from the additions of proteoses.[9] Moreover, pure proteoses in Tyrode solution have no effect on the growth of cells in vitro;[200] the addition of plasma to the medium has very little effect, but the addition of even small traces of embryo extract to cultures from fresh heart tissues produces

astounding results. The extract is not by itself responsible for the increased growth, since, for one thing, it is present in insufficient amount, and however great its concentration it would not produce such extensive growth. The stimulation therefore appears to be dependent on the mutual interaction of proteoses, embryo extract and plasma. Finally the action of the proteoses is clearly different from that of the extract, because the growth-promoting action of embryo extract is destroyed by heating the extract,[88] while proteoses still show intense activity after prolonged boiling. A conceivable explanation of some of these anomalies may lie in the idea that some enzyme in the extract acts on the proteoses to produce substances of smaller molecular weight at a very slow rate, which although they never attain a sufficiently high concentration to become toxic to the tissues are yet constantly providing minute amounts of nutritive substances. Perhaps amino-acids are thus enabled to exert their stimulating effects continuously, and so to keep the tissue in an actively growing condition. On the other hand it is difficult to reconcile this with experiments on the digestion, or increase of free amino-nitrogen, in solutions of proteoses caused by plasma and by embryo extract. Plasma digests the proteoses far more actively than does the extract. The actual products of digestion may of course be different, but the results suggest some other function than digestion as the reason for the properties of embryo extract. This view is also supported by the failure of proteoses to stimulate old strains of fibroblasts in the same way that they affect fresh explants.

(c) *Growth-promoting Substances, and the Causes of Cell Proliferation*

What then is known of the stimulating properties of embryo extract itself? Embryo extract is most active when made from very young tissues, and there is a considerable quantity of active substance which can be obtained by simple extraction with salt solution,

that is to say the tissue pulp can be extracted two or three times and the extract still displays growth-promoting properties. The extract when freshly made is a clear, slightly opalescent, fluid, which on standing even at 0° C. slowly deposits a precipitate. But the supernatant fluid remains active, even after removal of the precipitate. On the other hand, if the extract is kept for a long time it loses its growth activating powers, and loses them more quickly the higher the temperature. Heating to 56° C. greatly reduces the activity, and raising the temperature to 70° C. for ten minutes completely abolishes it. Moreover, filtration through a Berkefeld filter reduces the activity,[88] and it is said that there is no evidence for the passage of any active substance through a Chamberland filter. Shaking the extract sooner or later renders it inert. This may well be an oxidative change, since bubbling with oxygen is equally effective.[112] But neither denaturation of an active protein, nor denaturation of a protein followed by adsorption upon it of the active substance, are ruled out as possible explanations of this reduction of activity, and it is not impossible that the apparent inability of the active substance to pass through a Chamberland filter may be due to adsorption of the substance on the filter. In contrast to this apparent instability, the extract may be dried at room temperature in vacuo without loss of activity.[20]

The active constituent appears to be either a globulin or associated with a globulin.[92] Precipitation experiments, and partial separations of the constituents of the extract, mostly fail owing to the difficulty of removing the reagents used in the separation, which are frequently of a character which would be harmful to living cells. Consequently only such comparatively harmless reagents as alcohol, ether, CO_2, &c., can be considered. Active growth-producing powers are claimed for solutions containing the precipitate produced from embryo extract by CO_2, but attempts to produce further purification by reprecipitation, &c., have failed. The thermolability of the active constituent together

with its difficulty in passing through filters means that all operations have to be carried out aseptically, for the solutions cannot afterwards be sterilized. It is not surprising therefore that, as long as the only test for the activity of embryo extract depends on its property of stimulating growth in tissue cultures, research into the nature of its essential constituents will be a slow and unsatisfactory process, and one which will be full of pitfalls for the unwary.

One characteristic property of embryo extract is that it generally contains sufficient reduced sulphur or SH groups (30–40 mg. glutathione per 100 c.c.[19, 111]) to give a positive nitroprusside reaction, and in this connexion the light of understanding appeared to be shining for a time, and several more or less unrelated observations seemed to be linking up to give a corner of the jig-saw puzzle entitled "the process of growth." On the one side came information concerning the distribution and physiological activity of SH groups in animal tissues and extracts, on the other came news of the relationship between glycolysis, anaerobic conditions and active tissue growth. Were the two connected, or was it merely a coincidence that growing tissues produce conditions which favour lactic acid production and the occurrence of SH groups?

The theory has been put forward[100, 166, 173] that growth by cell division depends essentially on the presence of fixed SH groups in the immediate vicinity of, or more probably in localized areas within the dividing cells, and there is much evidence that indicates that such a view might not be unreasonable. The SH group is an active group, not only in connexion with tissue oxidations, but also it is capable of activating certain enzyme systems.[188] As protein solutions age, the protein molecule tends slowly to lose water and to denature,[146] and in the process of denaturation there is often an unmasking of SH groups.[102] Moreover it has been shown that the denaturation may be brought about by such substances as urea,[108] when these occur in fairly high concentration. A consideration of the

conditions within the dividing cell leads to several interesting speculations on these lines. In the first place there is much evidence for extensive and rapid changes of state in the protoplasm during the formation of spindles, asters, &c., and the two poles of the cell appear to be the centres from which such activity originates. It is perhaps noteworthy that the configuration of a spindle and asters can be artificially produced in a model composed of Indian ink particles suspended in a solution of potassium nitrate. If a drop of the ink containing potassium nitrate is placed on a layer of solution of this salt in rather higher concentration, and then on either side of this drop are placed two other drops containing a considerably higher concentration of salt but very little Indian ink, then the whole model goes through a series of changes which closely resemble in appearance those visible in a living cell during mitosis.[15] The ink particles arrange themselves in such a manner as to imitate the chromosomes and spindle configuration. Such a resemblance is of course only superficial, but it is at any rate suggestive that the essential rôle in the performance is played by the two regions of high concentration which represent the poles of the dividing cell. At mitosis, is there a sudden and localized accumulation of metabolites at the poles of the cell? If so, the changes in state of the protoplasm, with denaturation of some of the proteins, may be due to this cause, and the appearance of SH groups would follow. These groups then, once they are liberated, may play some further important part in the process. It is interesting therefore to study the effects of the presence of SH groups in and around cells, and to investigate their occurrence in rapidly growing tissues.

Reports from a school of workers in America [100, 101] showed that the addition of solutions containing SH groups, such as thioglucose, glutathione, cysteine, &c., were active in producing an increased growth of plant tissues, protozoa, and also apparently favoured the process of wound healing in animals. In the case of

plants it was stated that the increased growth in the root-tips, &c., was entirely due to the greater number of cells rather than to their increased size, or in other words that the stimulation of growth was produced by augmenting the division rate of the cells, i.e. favouring growth by cell division. It is well known that growth can be easily checked by the addition of small traces of lead salts, and it was suggested that this inhibition was due to the removal of the SH groups.

Further evidence in favour of the importance of the SH group in the growth process was adduced from the study of sea-urchin eggs.[174] Unfertilized eggs do not give anything more than a very feebly positive nitro-prusside reaction, if they even give that, whereas as soon as fertilization has taken place the nitro-prusside reaction is very strong, and of course cell division starts at a very rapid rate. Actual measurements of SH group content confirm this increase. The fertilized egg, just prior to division, contains 46 mg. of glutathione per 100 gm. net weight.[173] Moreover, if the eggs are treated with very dilute solutions of mercuric salts the division of the eggs is suppressed and the SH content is decreased. The rate of division can however be augmented again by the addition of such substances as cysteine to the water in which the eggs are living.

To return to tissues growing in vitro, it was found that as long as the embryo extract in the medium gave a positive nitroprusside reaction growth could occur freely, and that the cessation of growth was coincident with disappearance of a positive reaction.[70] It is important, too, to bear in mind the fact that the reduction of activity of embryo extract by heating, by shaking, or by standing for a long period, is consistent with the view that its activity might be dependent on the presence of SH groups, for these would quickly be oxidized and destroyed by such treatment, especially since the extract is generally slightly alkaline. Another possibility suggests itself. When embryo extract is allowed to stand, it slowly deposits a precipitate. If, as seems likely, this precipitate consists of denatured

protein, its continuous formation for several days might lead to an accompanying production of SH groups, thus perhaps aiding the growth of the tissues placed therein.

Further, peptic digestion of proteins unmasks SH groups from the molecules,[102] whereas tryptic digestion does not have this effect since it only takes place in neutral or slightly alkaline solution, and these conditions allow the groups to be quickly oxidized. It may then be significant that peptic digests of proteins are in the main more active in promoting growth than those effected by trypsin.

Now SH groups can only exist free under conditions of low oxidation potential, so that their existence in the animal body will be confined to those regions where such a state of affairs exists. It is interesting therefore to note that the three places where cell division is proceeding most actively in the adult animal, namely the lymph-glands, the Malpighian layer of the skin, and the testes, are all regions which are not well supplied with blood, that is to say that the blood-vessels do not come into anything like such intimate contact with the cells of these regions as they do in muscle, or in endocrine glands for example, so that it is possible that the cells of these regions are not very freely supplied with oxygen. Conditions of low oxidation potential may induce tissues to obtain their energy by anaerobic methods and consequently the high rate of anaerobic glycolysis which so characteristic of growing tissues, embryonic and cancerous, may not be unconnected with the presence of SH groups in the cells. All these different observations could then be taken to indicate the possibility of basing a theory of cell division on the occurrence of low oxidation potential within the cell, the presence of SH groups and the occurrence of anaerobic glycolysis. But unfortunately, on putting this theory to the test, it fails in several important respects.

First, with regard to denaturation, it may be mentioned that the process is occurring rapidly in the formation of the keratinous layer of the skin, and

probably also in the laying down of cartilage and the ground-substance of bone. It is true that the skin is a rapidly growing organ, but this is not necessarily true of the other tissues. Moreover, in vitro, keratinization and the formation of bone and cartilage are correlated with slow, rather than with rapid, proliferation of the cells. Keratinization is in fact often regarded as a sign that the medium has ceased to be suitable to the growth of the tissues. It is perhaps noteworthy that all actively growing tissues, normal and neoplastic, show a high water content, and in the process of ageing in an animal there is a constant drying up. As the proteins become less hydrated the rate of growth declines, and the highest growth rates occur when the proteins are at their 'wettest'. In some way therefore cell division may be correlated with the earlier processes of drying rather than with the later ones which lead eventually to actual denaturation. The loss of water from the growing tissues can apparently be checked, at least under artificial conditions, for, in vitro, tissues have been kept in the same embryonic condition of unrestricted proliferation for a considerable number of years.

If anaerobic glycolysis favours cell division, then tissue cultures might be expected to thrive under anaerobic conditions. Such, however, is not the case.[162, 191, 201] For a long time it seemed that almost the only normal tissues which showed any capacity for activity at all in such circumstances, were tissues from the heart of chick embryos of less than five days' incubation.[24] Certain cancerous cells show signs of life without oxygen if glucose is present in the medium, but not otherwise. Recently, however, it has been shown that osteoblasts (periosteal fibroblasts) have some capacity for anaerobic activity, especially if they are first grown in vitro in the presence of oxygen.[129] Other investigators, working on heart tissue under rather different conditions, do not however support these conclusions.[104] It may therefore safely be said that even if tissues can live anaerobically, such con-

ditions are not exactly very favourable to their growth. But before leaving the subject of anaerobiosis there are one or two effects of anaerobic conditions in cultures which should be taken into account. For example, an interesting experiment [91] was performed, in which tissues of different types were cultured in test-tubes at different depths in a plasma coagulum. The three types of tissue tested were fibroblasts, wandering cells, and carcinoma cells, and it was found that the carcinoma cells were only able to grow when very near the surface; the fibroblasts could be grown at rather deeper levels, while leucocyte colonies flourished at any depth. Cultures deep in the clot would doubtless very soon suffer more or less from oxygen lack, and be thrown back on anaerobic glycolysis for their energy supplies. The depth at which the various types were able to grow might then be taken as a measure of their capacity for anaerobic existence, but other factors are also involved. Anaerobic glycolysis produces lactic acid or some similar and probably harmful body, and the more anaerobic the conditions the greater will be the amount of this substance that will accumulate. Consequently in this experiment one of the factors which may stop the growth may easily be such an accumulation. The leucocytes grow as isolated cells far out into the medium, and they will therefore not only be able to obtain oxygen more easily, but also the diffusion away into the medium of any lactic acid or similar body produced will be relatively less difficult than it would be from the closely packed fibroblasts. This may be an important factor in increasing their capacity to grow at greater depths than can the fibroblasts. The carcinoma tissue on the other hand produces much lactic acid near its closely packed cells, so accumulates an excess very quickly, and is, under these stagnant conditions, very sensitive to oxygen lack. Such considerations show that differences in technique, which may allow of different rates of diffusion of metabolites, may account for some of the anomalous results which are all too frequent in this branch of the subject. On the whole,

however, it cannot be said that, even when the question of the accumulation of lactic acid has been eliminated there is any very strong evidence to suggest that anaerobic conditions favour the growth of tissues in vitro. They may allow it to occur, but they certainly do not encourage it. Stagnation conditions, it is true, are sometimes more favourable to growth than conditions where the medium is frequently renewed,[25] but this almost certainly depends on other factors besides the oxygen supply.

Now again, if the SH groups favour growth, it would be reasonable to suppose that their addition to tissue cultures would produce great stimulation in this respect. The results so far obtained, apart from the observations already mentioned on the SH content of embryo extract, have been negative or of very doubtful significance. The only apparently positive results were offset by the finding of an equal enhancement of growth by the addition to the cultures of comparable amounts of arginine in place of the SH-containing glutathione.[111] Incidentally the concentration of glutathione in embryo extract is not particularly high, and is comparable with that found in normal human blood.[19] The addition of substances containing SH groups, such as cysteine or glutathione to sea-urchin eggs was not found to produce any change in the division rate.[69] This in itself is not very convincing evidence, for in the first place the eggs already contain large quantities, and may be dividing at the maximum rate of which they are capable. It is also possible that in these comparatively impermeable cells the glutathione might not gain an entry, although this is perhaps unlikely since washing the eggs continuously is known to reduce their glutathione content and to lessen their powers of development. It may be significant that chick embryo extract, which will stimulate tissues of widely differing origin has no effect on the division rate of sea-urchin eggs. On the other hand, however, the addition of iodoacetate and fluoride ions,[69] which are known to inhibit glycolysis in muscle and yeast, produced no

significant effect on the division rate of sea-urchin eggs, and the iodoacetate ions apparently entered the eggs, since their reduced glutathione content was considerably lessened by such treatment. These observations then, on the whole, point to the conclusion that anaerobic conditions and SH groups are not of primary importance in the stimulation of division in these eggs, although some of the results are certainly of a somewhat contradictory character. Cyanide, which reduces respiration and not glycolysis, does on the other hand adversely affect the division of the eggs,[69] although it is true that the eggs are perhaps more sensitive in the resting stages than during the actual process of division.[150] This observation again contrasts with certain others [166] on very young chick embryos in vitro, in which the addition of sufficient cyanide to inhibit respiration to thirty per cent of its normal value, without affecting glycolysis, allowed the tissue to undergo normal growth and differentiation. But the very young chick embryo may be a special case, as is indicated by the survival of its tissues under anaerobic conditions.

The only conclusion which it seems justifiable to draw from the rather miscellaneous data in connexion with the energetics of growth, is that normally it is a process which involves a high rate of metabolism and consumes much energy, but the actual source of that energy is comparatively unimportant. A solution of chlorhydrate of quinine [172] in a concentration of 1/10,000 practically stops the growth of tissue cultures, and at the same time reduces the sugar consumption to sixty-six per cent of its normal value. This perhaps might be taken to indicate that anything in the tissue culture which interferes even slightly with the utilization of sugar for energy purposes has a checking effect on growth, although, of course, the quinine may act in other ways too. The developing chick has been shown [165] to pass through three stages at least in which the primary foodstuffs oxidized are in turn carbohydrate, protein, and fat, which again proclaims growth as independent of the energy source; and in addition to this it has

already been noted that in the very young stages of the chick, glycolysis or respiration may be almost equally effective. Finally it has been shown that practically all tissues growing in vitro produce lactic acid,[124] which is only partially checked by shaking with oxygen.[145] This might be taken to indicate that lactic acid and growth are closely connected, but on the other hand the amount of lactic acid produced is, as far as the evidence shows, more or less inversely proportional to the rate of growth.[56] To sum up, SH groups, conditions of low oxidation potential and anaerobic glycolysis must be regarded as frequent accompaniments of active growth, but almost certainly not as its cause.

The development of the sea-urchin egg opens up one other aspect of growth which is of interest at this point. During the first stages of its development there is practically no increase in the actual mass of the embryo, and all that goes on is a series of nuclear divisions and a parcelling out of the cytoplasm. In other words instead of a single cell with a nucleus there arises a large number of smaller units, which points to the need of making a fundamental separation between several processes involved in growth by cell division. In the first place there is nuclear division, which has previously been shown to be separable from and independent of cytoplasmic division, and now it seems possible to distinguish between a process involving purely cell division, and one involving growth as well. In most cases an animal develops, except in the formation of bone, &c., by increasing the mass of its protoplasm, which is concurrently partitioned off into small units by frequent cell division, so that growth and cell division are often regarded as synonymous, but in the sea-urchin they are clearly separable, for the increase in the amount of protoplasm does not at first keep pace with the rate of cell division, and so the cells get constantly smaller. It is important then to keep this distinction in mind in questions relating to growth-promoting substances, and there is always the possibility that although as a general rule increased protoplasmic

growth is followed by cell division, yet the two may be independently stimulated.

Attempts have been made on tissue cultures to establish a definite ratio between the nuclear and cytoplasmic masses. Although there is no doubt that in any given type of tissue, as for example is clearly demonstrated in a tissue culture of epithelium, the cells are remarkably uniform in size and have nuclei occupying a definite proportion of the cell, yet attempts to express this constancy are apt to lead to fallacious results.[14, 59] The weights of nucleus and cytoplasm can manifestly not be obtained, and only rough estimates can be made of their volumes. Moreover there is the difficulty of deciding how much is to be included in the cytoplasm. When cells are placed in a very hypotonic medium the cytoplasm becomes filled with vacuoles. In other media it may become laden with fat, and if reliance is placed on measurements of volume both these conditions will lead to great changes and variations in the value of the nucleo-plasmic ratio, for quite obviously the only valid ratio is between the actual amounts of nuclear material and of true cytoplasm without any of its adventitious inclusions. Some ratio based on biochemical data, as between the amount of nucleic acid, an essentially nuclear constituent, and the total protein of the cell,[51] or between purine nitrogen and total nitrogen minus purine nitrogen,[165] probably offer the most hopeful means of establishing a useful ratio, but at present the applications of such methods are very limited, and must remain so until more delicate micromethods can be evolved. From a superficial examination of various cell types it seems quite obvious that in each type of cell the nucleus can, as it were, look after and control a certain definite amount of cytoplasm, which, if it could be satisfactorily measured, would be a very sound way of distinguishing various cell types.[130]

It is quite probable, in fact almost certain, that cell division and growth are dependent on a series of conditions for their fulfilment and there is no one factor which is responsible for growth by itself, although at

any one time one particular condition or substance may determine by its presence or absence whether growth shall or shall not occur. There may be factors of a more or less nutritional character which allow new protoplasm to be formed, and probably this is where the utilization of proteoses is important. Again there seem to be substances, or conditions which lead to intense cell proliferation, as for example when embryo extract is dialysed through collodion membranes into physiological salt solution and the dialysate is found to cause considerable numbers of cell divisions in cultures to which it is applied.[202] A solution with similar properties can be obtained by dialysing the yolk of incubated eggs in the same way,[204] and this dialysate also produces intense cell multiplication when fed to heart fibroblasts in culture. The whole yolk inhibits growth. It has not been found possible to maintain tissues alive for long [6] in the dialysate from the embryo extract, which suggests that the cells are definitely lacking some other important constituent which is normally supplied by the whole extract. At this point an examination of the growth behaviour of tissues in certain other media may be undertaken with advantage. It has already been noted that in plasma from an adult bird the tissues of the chick embryo survive for several days and display but a limited amount of migratory activity, and that the high growth rate of the embryo is not maintained. If the culture is periodically fed with heparin plasma the tissues are not starved and may be kept alive for very long periods of time but without growing to any appreciable extent. Of interest then is the question as to why the cells remain without growing. Are they being actively inhibited, or are they lacking a stimulant for growth? The tissues of the adult animal, with one or two exceptions, are certainly not growing and they are being bathed by a medium which is very similar to, and which is in equilibrium with, the plasma. Is the cessation of growth of the adult due to inhibition by some substance in the plasma, or are the cells of the adult lacking some neces-

sary stimulant which is present in the plasma of the young animal, but which progressively disappears? These are questions which must still remain unanswered, but there are certain parallel experiments on serum which may throw light on the subject. Serum essentially differs from plasma in the fact that fibrinogen and prothrombin have disappeared, and thrombin has taken their place, the fibrinogen having been removed in the clot as fibrin. As a medium for tissue-culture experiments serum has been used with varying results.[37, 49, 58, 200] At one time it was claimed that the serum from adult birds was definitely inhibitory to the growth of fibroblasts while that from younger birds was less so. This was correlated with a slight change which was found in its constitution.[8, 39]. It was believed that it contained a growth-promoting substance, which was precipitable by CO_2, and of a protein-like nature, and also an inhibitory substance which was fatty or lipoidal in character. As the age of the bird from which the serum was taken increased, so the protein body became less in amount, and the lipoid substance more. The increase in the amount of the inhibitors was regarded as more important. The results have not been confirmed, and in the light of other work on the effects of serum on cells in vitro, would well repay further investigation. Many workers find that serum is not inhibitory, but on the contrary, that it often leads to quite a marked, if temporary, outburst of growth. Undoubtedly the growth-promoting properties of serum, plasma, and heparin-plasma, are all slightly different, but in the present uncertain state of knowledge concerning the whole process of blood coagulation, it is a little dangerous to speculate as to the particular parts played by each constituent of the 'fibrinogen-thrombin complex'. There is a certain amount of evidence [206] that prothrombin favours the growth of tissues and that it is antagonized in this respect by heparin. But until the relative distributions of prothrombin, thrombin, fibrinogen, and possibly anti-thrombin in plasma coagulated in various ways and in serum, are made more certain,

any definite conclusions on the subject would be unjustified. Moreover, the manner in which heparin acts to prevent the coagulation of plasma is of fundamental importance, and if as recent work seems to show [160] it prevents thrombin from acting on the fibrinogen, and not by preventing prothrombin from becoming thrombin as at one time supposed,[109] then some of the evidence in favour of the growth-promoting powers of prothrombin must fall to the ground. Heparin itself exerts no direct inhibiting action on the cells, but it seems to reduce the growth-promoting powers of embryo extract, plasma, and of serum : in the last case, however, growth although not so rapid is allowed to proceed for a longer time than in serum without heparin.

The whole problem has quite obviously not yet been sufficiently thoroughly investigated, and it again is one worthy of further study, though it is also one which is crowded with difficulties owing to the inherent variability of the plasma and serum. Although the constancy of the internal environment of the higher animals is well recognized, the slight differences, to mention but a single instance, in endocrine content of the blood or serum of the same animal at different times is enough to account for many anomalies in its growth-producing power.

Again in the usual preparation of serum, the fluid remains in contact with the coagulum for a considerable time, and in that coagulum are cells, red and white, so that it is possible that those cells may materially alter the growth power of the serum. Indeed, there is much evidence which at any rate suggests that the white cells may have considerable power to do this.[33] In fact, extracts from the buffy coat of blood, which contains a very high proportion of white cells, have been made and found to stimulate the growth of fibroblasts. Cultures of leucocytes or wandering cells, though not actually derived from the buffy coat but from the spleen, when placed near cultures of poorly growing fibroblasts [43] have been found to stimulate the latter

to renewed activity, and the theory has been put forward that in the healing of wounds in the body, one of the functions of the aggregations of white cells is to provide 'trephones' or nutritive substances upon which the fibroblasts may feed, and so be able to multiply and form the fibrous tissue so constantly associated with wound-healing. The nature of these 'trephones' is entirely unknown, and the name has simply been coined on the supposition that the white cells act on the serum or plasma to produce nutritive bodies upon which the fibroblasts feed. Beyond the fact that wandering cells can utilize plasma for their continued growth and multiplication whereas fibroblasts cannot do so, and the fact that the proximity of similar cells from spleen cultures increases the growth activity of fibroblasts there is very little evidence for the actual existence of 'trephones' as discrete bodies. If, as was projected above, fibroblasts depend for growth of new protoplasm on a supply of amino-acids in low concentration, then leucocytes may provide this, as they are known to contain proteolytic enzymes.[188] On this theory it is necessary to suppose that fibroblasts in a plasma medium obtain just sufficient amino-acids for maintenance purposes, but they cannot add materially to their protoplasm without external assistance.

Before attempting to summarize and, if possible, to sort out this bewildering confusion of observations on so-called growth-promoting substances, it is still necessary to enumerate several other substances which have at one time or another been hailed as significant aids to the growth of cells. They mostly come from the realms of tissue extracts. Of these may be mentioned as of primary importance extracts of spleen, bone marrow, and Rous sarcoma of the fowl.[92] The mode of action of the last is completely obscure, but probably the spleen extract acts in the same way as extracts of blood leucocytes—after all, they are virtually the same things. Extracts of other organs often produce minor accelerations of growth in cultures but nothing significant, Liver extract is said to inhibit growth.[189] Possibly this

inhibition may in some way be caused by the heparin in the liver which has been shown to reduce the growth-promoting activity of embryo extract,[206] although itself it is relatively harmless to the cells. Extracts of thyroid gland have a slight stimulating effect, and it has been claimed [92] that, when thyroid tissue is grown adjacent to colonies of fibroblasts [67] in vitro, the growth of the latter is stimulated for a few days, but that soon the thyroid cells become encapsulated by fibroblasts and the stimulation ceases. At any rate it may safely be concluded that neither thyroid extract, nor in fact any other normal tissue extract, except perhaps that of spleen, has anything comparable in growth-promoting power to embryo extract. Certain observations were made on extracts of yeast,[105] but the results were rather inconclusive; and the evidence that any of the known vitamins affect the growth capacity of cells in vitro is lacking, although a theory has been advanced that vitamin B stimulates epithelial growth,[105] but leaves fibroblasts unaffected, and another theory that vitamin B is the much looked for growth factor for all tissues.[26]

Finally there is the subject of carcinogenetic tars, and the derivative polycyclic hydrocarbons. These substances are definitely capable of producing neoplasms in which cell division and growth are both progressing rapidly. The growth is pathological, and perhaps is not strictly comparable with normal growth, and certainly the requirements of cancerous cells are different from those of normal ones, but here are definitely substances which lead to increased growth. So far they have not been critically tested on normal cells in vitro, but what evidence there is suggests that they do not behave like embryo extract in causing a direct stimulation of growth, but rather do they slowly alter the character of the cells in such a way that they can afterwards live and proliferate on serum alone.[122] Fibroblasts normally cannot proliferate rapidly in serum or plasma; wandering cells can. The action of these polycyclic hydrocarbons may then turn out to

be similar to an alteration in character of the fibroblasts to render them in some respects more like wandering cells. Direct evidence on the exact mode of action is not yet forthcoming, but it appears to be rather different from that of embryo extract, and therefore the growth produced by it is not strictly comparable with embryonic growth. Certain experiments on the effects of carcinogenic substances on the development of chick embryos are interesting and will be mentioned in connexion with problems of differentiation.

Is it now possible to piece together from the vast mass of experiments and observations which have been made on so-called growth-promoting substances, and of which many are sadly uncontrolled or biased in some particular direction, any connected hypothesis as to the nature and mode of action of these fascinating bodies? The chaos has mostly arisen owing to slight variations in technique employed and an inadequacy of definition in the statement of results.

In the first place the particular tissue whose growth is under investigation is of extreme importance. As far as embryo extract is concerned the cells known as fibroblasts derived from mesenchyme cells in the body are all stimulated to a greater or less extent; though even here the actual concentration of the extract which produces the maximum effect differs somewhat according to the origin of the tissue. In future investigations it would be well to confine attention primarily to one particular type of fibroblasts, say from chick heart, or periosteal tissue. Some tissues are definitely inhibited by large quantities of extract, as for example are wandering cells,[45] so that even if the active substance in extract is discovered the problem of growth is by no means solved. In the remarks which follow attention is only being given to the problem of growth-promotion in cells derived from chick heart or chick periosteum. Although chick embryo extract appears to have similar effects on tissues from many sources and is not species specific, being equally able to stimulate rat fibroblasts, for example, yet in the existing confusion it would be

as well to confine the discussion as far as possible to one tissue type.

Is embryo extract in itself sufficient? In other words does it provide all the substances necessary for continued growth? When present alone in hanging-drop cultures it allows a copious outgrowth of cells from the original explant, without any addition of plasma, but attempts to continue the life of such cultures have all in the end been unsuccessful. There are many possible explanations for this failure, but there is no single one to which it is possible to attribute it. However, a mixture of embryo extract with plasma is a sufficient medium, and in it cultures will grow indefinitely. The plasma however has been said to play no part in this growth except that of providing a mechanical support for the cells; though upon what grounds it is denied any growth-producing activity so categorically, it is not clear, as plasma alone can maintain tissues alive for long periods, although they certainly do not grow.

Embryo extract alone, of all tissue extracts, is capable of exerting these great growth-promoting properties. The effects of spleen extract, leucocyte extract, and extract from Rous sarcoma are on a smaller scale. The growth-promoting substance (with which will, in what follows, be understood substances or conditions) must therefore be present in an active form in these extracts and in these only, on which grounds it would seem possible to exonerate thrombin, prothrombin, thrombokinase, and any other substance related to blood clotting from this specific effect of embryo extract. This is not to say that under certain conditions such bodies may not have some power of causing increased growth of tissues, but they are manifestly not the responsible agents in embryo extract, because they are or may be equally present in other tissue extracts. Against this may be argued that in other extracts inhibitory substances are also present. This merely reverses the problem into one in which it is necessary to determine what are the substances which are present

in most tissue extracts which inhibit growth; growth then must be regarded as a process which can only occasionally escape from the clutching hand of inhibition, and embryo tissue, spleen, and leucocyte extracts as the only substances which do not contain the inhibiting agents. On the face of it, this looks like putting the cart before the horse, and until it is demonstrated that that is the only way in which the cart will move, the more orthodox arrangement would seem preferable.

Simple conditions like hydrogen ion concentration, buffering power, and inorganic ionic content generally can be ruled out from the main question since there is nothing peculiar to embryo extract in these conditions. Inorganic ions often produce proliferation in the animal body, but in vitro there is no evidence that either as such or by their effects on the osmotic pressure of the medium do they affect growth by cell division in any way except adversely.

Anaerobic conditions although frequently accompanying growth are not causative agents and similarly glutathione content and SH groups generally are not responsible.

The extracts which are active are those from tissues which are highly nucleated, so that some nuclear constituent may well be responsible; but additions of thymus nucleic acid and its salts,[9] although capable of producing slight increases in growth under certain conditions produce nothing like the remarkable effects of embryo juice. Consequently although nucleic acid may play a minor part, it is not of first importance.

Proteoses, in combination with extract, produce remarkable results, but alone they are not of any value to cells. This looks like a signpost but the writing on it is in code, and much work will have to be done before it can be deciphered. The activity of the extract is not due to proteoses; they are not present in any appreciable amount, and also they are thermostable, whereas extract is definitely reduced in activity by heat. The general instability of embryo extract forces comparison with enzyme systems, and putting two and two

together to make a rather dubious four, the suggestion emerges that the enzymes have something to do with proteoses. Enzyme action is further indicated by the extremely small quantities of extract which are necessary to cause considerable growth particularly in the presence of proteose solutions. What enzymes therefore are present in embryo extract? This has recently been investigated and the results are interesting.[19] The extract was made according to the normal procedure used in tissue culture, namely by grinding up the embryo in Tyrode solution, and allowing it to stand for about thirty minutes, before centrifuging away the solid particles of the crushed embryo. Under these conditions there is no protein-splitting enzyme extracted, although these may be obtained from the same tissues by glycerine and acid extraction, but on the other hand there are present a polypeptidase and, in greater amount, a dipeptidase. In addition to these there is an amylase and a lipase. The polypeptidase shows an optimum hydrogen ion concentration for its effectiveness at *p*H 7, without much decrease to *p*H 6 or 8, while the dipeptidase has a sharp maximum at 8·5. Similar dipeptidases are found in human spleen, kidney, and many other tissues, though often with an optimum slightly less on the alkaline side. The presence of these enzymes in other tissues weakens the whole case, since other extracts have much less growth-promoting power. However, there is one significant fact which emerges, and that is that in the extract there are two enzymes capable of acting on breakdown products of proteins, and together with the proteolytic enzymes in the plasma they may in some way be partially responsible for the effect of the extract in making proteose solutions available to the tissue. In growing tissue, however, it is important to consider protein synthesis as well as protein breakdown, and some interesting information might be forthcoming by further investigations along those lines. Has embryo extract any particular properties which would aid in synthesis of proteins?

The way therefore in which the tissues obtain their

nitrogen and build up from it their new protoplasm still remains entirely mysterious, and it must be confessed that though it is possible to maintain tissues in an active state of growth for decades, under more or less artificial conditions there is no real understanding of the mechanism of growth, and there still remains an absorbing problem, whose surface has been only slightly scratched, awaiting the investigator who can come to it with entirely new ideas. At this present time the only conclusion to which it seems possible to arrive is that the circumstances provided by mixtures of embryo extract and plasma are such as to lead to great activity and growth on the part of the tissues, but it is impossible to specify any one substance or condition which can be regarded as a key. Only further research by very carefully controlled experiments can point to those substances or conditions which are of greatest importance.

ORGANIZED GROWTH

DIFFERENTIATION AND CELL TYPES

(*a*) *Introductory*

SO far have been discussed the conditions of culture which lead to the survival of tissues in vitro and to the unlimited proliferation of their cells. But there is another and very important application of tissue culture methods, and that is towards the second aspect of the growth problem, namely the differentiation of normal tissues and the assumption and performance of the functions of the different kinds of living cells of the animal body. A normal organism grows and becomes fully developed by the combined working of several distinct processes. One of these, growth by cell division, has been discussed in so far as tissue culture methods have any bearing on the problem; but no organ grows only by cell division: sooner or later the cells specialize to perform their proper functions, and generally, although probably not always, coincident with that specialization cell division ceases. Broadly speaking, differentiation and growth by cell multiplication are quite separable, and are mutually opposed to one another. A functioning cell rarely divides although in a developing organism one cell may be functioning and its next-door neighbour still dividing. Now the functions performed by cells are vastly different, and the first question that leaps to mind in considering differentiation is as to what are the factors which operate to produce a functioning cell out of one which is apparently of quite general type, and whose sole occupation appears to be growth and division. Tissue culture affords an opportunity of isolating groups of cells from various

organs at any desired time in their development, and of investigating their behaviour under artificial conditions.

Embryonic cells undoubtedly have great inherent capacities for development along definite lines, but generally for the manner in which those potencies emerge they are dependent to an even greater extent upon influences external to themselves. The results obtained by tissue-culture methods together with those from other fields of experimental embryology have combined to shed more than a glimmering of light on the mechanism by which normal harmonious development occurs.

Before proceeding further it is necessary to define more precisely what is meant by various terms. Of these, differentiation may come first. A fully differentiated cell is a cell which has the necessary structure, mechanical, physical and chemical, to enable it to function. A typical mesenchyme cell or fibroblast, such as occurs in a culture from periosteal tissue of the chick in a state of unorganized growth, will not under those conditions form bone, although under certain other conditions it might be capable of so doing. It is therefore obviously not fully differentiated. On the other hand, as far as has been ascertained up to the present time, it is quite impossible for that cell to start to function as one of the rods of the retina of the eye, or as any type of epithelial cell for that matter. Although it may have several ways in which it might develop, it has already lost some of its potencies, and the capacity to become an epithelial cell of any sort is one of them. It is partially differentiated. Again, even in rapidly growing colonies of such mesenchyme cells or fibroblasts there are laid down between the cells fibres which stain by silver methods (argyrophil fibres), [18, 132] and as this may be considered as a typical function of true fibroblasts, some cells in the colony at least are functional, or perhaps all the cells are functional in this manner but not as true bone cells. If this latter conception is correct, and indeed even if it is not, it is necessary to assume that there are degrees of differentiation. The fibroblast is manifestly more differentiated than, for instance, a blastomere

from the eight-cell stage of a sea-urchin, which may develop into any part of, or even into a whole adult. This condition of partial differentiation is often conveniently described as determination, that is to say that the potentialities of the cell are determined but they have not been fulfilled. The use of the word determine in this connexion is perhaps unduly extending its meaning, which was initially intended to designate on purely embryological grounds the condition of cells which by virtue of their previous history had had their future mapped out for them, although they had not yet attained their full development. For example, the cells of the epidermis which at one stage overlie the developing optic vesicle in certain amphibia become influenced by the cells of the vesicle, in such a manner that to whatever spot on the epidermis of the animal they may be subsequently grafted they always develop into a lens, which would have been their fate if left in their original position. Such cells are therefore said to be determined for lens formation, and the idea is inherent in this term that no matter what conditions are offered to those cells the only way in which they may develop further is towards the construction of a lens. Determination is therefore an irreversible process. A similar term, and one which is perhaps more applicable, is chemodifferentiation.[113] This implies that some change has occurred in the physico-chemical make-up of the cell which causes determination, and ordains the future character of the cell. Chemodifferentiation is normally followed by histodifferentiation when visible differences occur in the cell so that they produce the characteristic appearance of the tissue to which they belong. In a whole animal histodifferentiation is normally followed by auxanodifferentiation when the organs assume their full size and proportions. Auxanodifferentiation is a complex of several mechanisms. Chemodifferentiated cells are still increasing in number, more and more are histodifferentiating, and these more mature cells are often enlarging their cytoplasm, or laying down ground substances of one sort or another, so that the organ as a whole may increase

greatly in mass during this third phase of development. Tissue-culture methods have been able to throw considerable light on these three processes involved in differentiation and on their relationships to each other.

It is comparatively easy to decide when histodifferentiation has occurred, but not so with chemodifferentiation, for in the latter process there are no visible signs accompanying the changes occurring within the cell. The changes which take place in chemodifferentiation are physico-chemical or metabolic, and until suitably sensitive microchemical tests can be developed must depend for their detection upon indirect methods of investigation, and tissue culture has been successfully used in this connexion.

Some of the early observed and now commonplace facts about cells living in vitro are of fundamental importance from the point of view of the problems of differentiation. As mentioned previously it is only possible to distinguish by their visible structure three types of cell when growing freely in vitro, epithelial cells, mesenchyme cells or fibroblasts, and wandering cells, and even among these distinction is sometimes difficult, if not wellnigh impossible, but the cells have certain characteristics of growth which aid in their separation. They may with profit be shortly reviewed.

Epithelial cells grow out as sheets or membranes, the edges of whose cells are all touching, and often joined firmly together so that if the membrane is torn it may break not at the cell boundaries but actually through the cytoplasm.[47] The cells tend to creep along surfaces and the best growth of epithelium occurs on the surface of a plasma clot where it meets the glass, the fluid phase of the medium, or the air. When planted in a coagulum the growth is said to be not so free, and membrane formation tends to be inhibited,[184] the cells becoming more spindle-shaped. A rather characteristic type of behaviour for epithelial cells is the formation of more or less hollow tubular structures [86] as outgrowths from the central implant; this occurs particularly when the implant is embedded in the medium. Often, and par-

ticularly when the medium is in some way unsuitable to their growth, epithelial cells undergo a characteristic change which is known as keratinization, in which a horny substance is laid down in the protoplasm of the cells. It is apparently a change which is exactly comparable to that which occurs normally in the outer layers of the skin. Cells with the properties thus outlined can be obtained from the skin, the lining of the alimentary canal, the thyroid gland, liver and many other sources. In other words epithelial cells as a whole have these properties in common when explanted into cultural conditions. There are ways in which their behaviour can be modified, but these must be reserved for discussion later.

Mesenchyme cells or "fibroblasts" grow typically as spindle-shaped cells which extend radially into the medium and form a network of cells as contrasted with the epithelial sheet. The appearance of the individual cells and some characteristics of their growth have already been described. Among the cells in the centre of the culture and extending outwards for some distance towards the periphery are nearly always to be found the argyrophil or silver-staining fibres which were mentioned earlier. In ordinary connective tissue in the body there are usually three sorts of fibre, argyrophil, collagenous or white fibres, and yellow or elastic fibres, and it is thought possible that the argyrophil fibres are a stage in the development of the collagen type. In vitro, collagen fibres as such, which typically stain with acid fuchsin and Mallory's stain, are seldom formed, and only in older cultures,[151, 153, 155] but the argyrophil type is very characteristic,[18, 132] and the formation of these fibres is as much a property of the mesenchyme cell culture as the keratin is of epithelial cultures, and affords some justification for the name fibroblasts which is habitually given to the mesenchyme cells which grow out from fragments of heart, periosteum and many other types of tissue.

Exactly how the fibres are formed by these cells has not yet been determined.[18, 131, 151] The true type of

fibre is only found in the immediate vicinity of cells, although it may be actually formed from the constituents of the medium. The argyrophil and connective tissue fibres are probably not simply modified fibres from the fibrin network of the plasma coagulum, as was at one time supposed,[2] although the presence of existing fibres facilitates the production of new threads.[151] Some investigators [133] have shown that fibres are actually secreted within the protoplasm of the cells, but whether when the cell moves on it leaves these fibres free in the medium as argyrophil fibres is doubtful. These intracellular fibres are probably of a different character. Others [132] consider that the whole process takes place in the medium, but in immediate contact with the cytoplasm, and that the cells are responsible for initiating the process, which cannot take place in their absence, or in the absence of substances diffusing from the cells into the medium.[60]

The third type of cell which behaves in a demonstrably different manner in cultures in a state of unorganized growth is also a mesenchyme cell, the so-called wandering cell. Such cells can be obtained from cultures of the white cells of blood, from various blood-forming organs, and a large number are often to be seen on the periphery of ordinary mesenchyme or fibroblast cultures, particularly in those from periosteal tissue. They are large, fast-moving cells, which have a much more independent character than either epithelial cells or fibroblasts. Whereas epithelial cells are generally in actual contact, and fibroblasts form a compact colonial network whose cells do not separate far into the medium, wandering cells behave much more independently, and completely isolated cells wander away into the medium. This they do by a very rapid motion of lamelliform pseudopodia. As such cells are watched under the higher powers of the microscope they may actually be seen in a state of active movement. The rounded pseudopodia are rapidly extended this way and that, and often show undulatory movements. The course of a wandering cell is erratic, whereas fibroblasts move more steadily outwards with a

regular gliding movement brought about by the extension of pointed pseudopodia from the anterior end. Although wandering cells are apparently much more independent of each other than fibroblasts, it is said that colonies planted adjacent to each other in a coagulum do not fuse together but preserve always a small neutral zone between them.[38] Fibroblast colonies on the other hand rapidly unite and their cells intermingle. Another important distinction is in their behaviour towards the medium. Fibroblasts for unlimited growth require considerable concentrations of embryo extract, but wandering cells can multiply freely without any or with only minimal amounts.[45] All they require is serum or plasma, and as was mentioned previously they have the capacity for making fibroblasts grow more actively, and for causing these cells to grow in media which are normally not adequate for them. One of their properties which may determine much of their behaviour is their capacity for causing a liquefaction of the plasma coagulum in which they are growing. Leucocytes of the blood contain protein-splitting enzymes so that the process is probably one of digestion. Many forms of cancerous tissues also have these powers of liquefying the medium, and in them they are often developed to a remarkable degree.[92] An incidental point which is not without interest is that a possible explanation for the sudden cessation of growth of colonies of fibroblasts may be concerned with their content of wandering cells. It quite often happens that fibroblast colonies cease to grow actively and die without any apparent reason and it is possible that the usual slight contamination of these colonies with wandering cells may be very important to their existence. Since these cells migrate outwards to the edge of a culture they tend to get lost after repeated sub-culture when only the more central parts are preserved, and when their numbers become too much reduced the culture may suffer in consequence. Many investigators believe that fibroblasts and wandering cells are more or less interconvertible,[17, 45, 52, 54, 90, 152, 154, 161] and that both are to be regarded as different functional states of the same

cell. They are manifestly closely related mesenchyme cells, but even if they are interconvertible there seem to be sufficient grounds for distinguishing them as separate types of cell in cultural conditions, for again, in certain cultures from the buffy coat of blood, tubular capillary-like structures occasionally develop, and this is presumably a modified form of the wandering cell approaching the epithelial type of growth.[110]

It is not possible therefore to distinguish with any certainty more than three types of cell in vitro from their appearance and behaviour when undergoing unorganized growth, but in reality there are within these three types probably an almost infinite number of different kinds of cells. This can be brought to light by so modifying the conditions of culture that growth ceases to be unorganized and the formation of various tissues occurs. For this purpose there are no golden rules; the differentiation of tissues occurs under a different set of conditions for each type of tissue, and in the process the inherent capacities of the cells are under the influence of environmental conditions. For the end result both are responsible.

Fibroblasts, epithelial cells, and wandering cells show definite evidence of a primitive histodifferentiation, and latent in each type there is strong evidence for considerable chemodifferentiation. For example, cells from the thyroid gland [68] may be grown as an epithelial membrane on the surface of a coagulum and show no signs that they are in the least differentiated into any special type of epithelium, but evidence has been adduced which shows that if caused to grow in a solid coagulum the cells form themselves into glandular structures which secrete a substance resembling the colloid found in the alveoli of the normal gland. More recent work,[55] however, makes it doubtful whether the cells do in fact ever form any fresh colloid in vitro, and it is likely that when the culture is embedded in the medium the original colloid persists for a very long time. It would indeed be rather remarkable if new colloid could be formed from such a very limited quantity of medium as is normally

present in a tissue culture. Moreover, thyroid alveoli [68] and kidney tubules [61] are stated to have developed from sheets of thyroid and kidney epithelial cells under the influence of fibroblasts added to the cultures, but again the interpretation of the results has been called in question.[55] Probably more convincing than either of these experiments are some which are obscure in method but striking in result. When cultures of the embryonic intestine [183] of the chick are made, both epithelial and

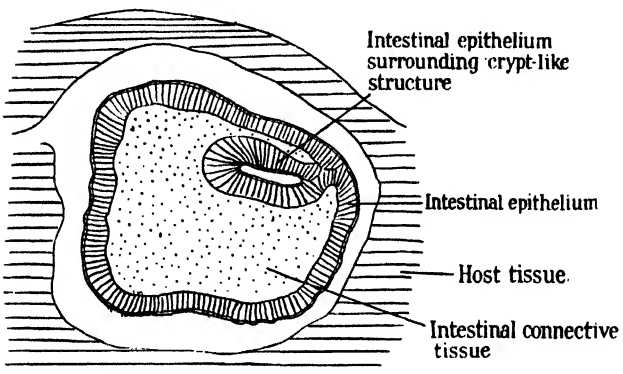

FIG. 8.—Diagrammatic section through an ' intestinal organism ' cultured in the lens cavity of the eye of a chicken from pure cultures of intestinal epithelium and fibroblasts. (After Törö.)

fibroblast cells emerge, and by suitable methods it is possible to separate the growths so as to obtain ' pure ' cultures of either type. Both can then be maintained in vitro in an active state of growth without differentiation. Now it has been found that if the lens is removed from the eye of a very young chicken, the cavity so formed becomes a suitable culture chamber into which tissues may be explanted; and at once the interesting problem suggests itself as to what would happen if various types of cells were grafted into this natural culture chamber. The lens is an epithelial structure.

Would any or every epithelium grafted into the cavity therefore form a lens ? The pure cultures of epithelium from the intestine were tried, but with completely negative results, in fact the epithelial cells degenerated and were resorbed. The pure cultures of fibroblasts behaved similarly as might have been expected. But, and here is the interesting phenomenon, when both types of cell were grafted together into the eye cavity, they did not degenerate but combined to form definite little growths in which the structure of the normal intestine began to make its appearance. The epithelial cells formed membranes round the mesenchyme cells and developed crypt-like structures with typical glandular cells. The gross anatomy was at fault, which was not surprising under such conditions, but the histological differentiation was a very fair representation of the normal. There are several important implications arising from this work. In the first place the apparently indifferent epithelial cells of the cultures of the intestine are in reality not in the least indifferent, but actually are completely chemo-differentiated and determined. They cannot be induced to form lens tissue, and the only way in which they apparently will develop is to form intestinal epithelium. For this purpose they appear to have all the necessary equipment and require only the appropriate conditions of growth, and one of these conditions is the presence of fibroblasts in their immediate vicinity. In this experiment they were fibroblasts from the intestine ; it would be interesting to see if any type of fibroblasts would be equally effective. The parallel case has already been mentioned in which the mixture of fibroblasts with epithelial tissue was effective in causing tubules to develop from kidney epithelium. But here there was some doubt as to the interpretation of the result since epithelial cells have a tendency to form tubular structures as a normal method of growth. Another point of great interest in connexion with the development of the grafts within the lens cavity concerns the mode of action of the fibroblasts. The facts of experimental embryology suggest again and again that there is a very strong

mutual interaction between cell and cell, and that one class of cells can induce a definite type of behaviour in another group. As an example of this, though taken from another field, may be given the original case which was first made clear. In the developing amphibian embryo the cells which invaginate below the dorsal lip of the blastopore induce in the cells overlying them the development of a neural plate from which the nervous system of the adult eventually develops. That is to say that cells which, though at the time still themselves indifferent in character, are destined to develop into alimentary canal, notochord, &c., can alter the behaviour of ectodermal cells in their immediate neighbourhood and cause them to start development along one particular line, namely to form nervous tissue. The same principle is involved as in the development of the lens of the eye of amphibia to which reference has already been made. The question therefore arises as to whether the fibroblasts in the intestine act as inducing agents for the formation of glands, &c., from the epithelium, and if so whether any form of epithelium would prove suitable, or whether only epithelium from the intestine would behave in this way. As far as there are at present any indications, the situation would seem to be that probably the cells of both types are irrevocably intestinal, and that successful experiments could only be performed with intestinal epithelium and fibroblasts from the same source. Mutual interaction of the two cell types causes the appearance of histodifferentiation from cells already chemodifferentiated. The future alone can tell.

(*b*) *Bone and Cartilage*

Before considering further the uses to which the tissue culture technique has been put in investigating the manner in which one type of tissue may induce a special type of behaviour in other groups of cells, it will be appropriate and helpful to review some of the work which has been done on the behaviour of two particular types of cells, namely bone and cartilage cells in vitro, for these cells illustrate many interesting phenomena in

connexion with the processes involved in differentiation. For example, in the normal development of the bones of the limbs, cartilage is first laid down in their place and this cartilage is surrounded by a two-layered membrane composed of fibroblasts, in the true sense of the word, on the outside, and of osteoblasts on the inside. These two layers of cells are visibly distinct and are fairly characteristic in behaviour. Together they form the perichondrium, or as it is later called, the periosteum. The osteoblasts are thought to be the cells which together with the cartilage cells are concerned with bone formation, the fibroblasts acting only as a covering and for the attachment of tendons, &c. When bone formation is proceeding, the cartilage cells become greatly enlarged (hypertrophied) and appear in large cavities in the cartilaginous matrix (chondrin). In this condition they contain the enzyme phosphatase which hydrolyses the phosphoric esters of the lymph and thereby leads to the deposition of calcium salts. The end result is calcified cartilage, which, although not unlike, is actually different from the bone by which it is replaced, and in which the calcium salts are laid down on a rather different fibrous organic matrix known as ossein. This matrix is the product of the cells (osteoblasts) originally forming the inner layer of the perichondrium. They also contain phosphatase, and can be seen to penetrate into the substance of the cartilage as the cells of the latter hypertrophy, thus replacing the cartilaginous matrix by bone. Now the interrelationships of these three types of cells, fibroblasts, osteoblasts, and chondroblasts (cartilage cells) form a fascinating corner of experimental histology. In the long bones of birds the whole of the original cartilage rod is removed from the centre of the shaft of the bone and in the fully formed structure only bone immediately underlying the periosteum is left, the cavity being entirely filled by blood-forming tissue known as marrow. The original cartilage rod is thereby converted into a tubular structure of bone, except at the two ends (epiphyses) where cartilage, or cartilage irregularly replaced by bone, persists. Bone formed by replacement

of cartilage is known as endochondral bone. The bone forming the shaft, although originally partly endochondral, is in the adult bird entirely endosteal, that is to say osteoblasts only are responsible for its development.

In the very early developing limb the whole cartilaginous area is present as a nodule of undifferentiated cells. Now if such an embryonic limb-bud is removed from an embryo of three days' incubation and cultured in vitro by the watch-glass technique,[80] in such a way that extensive uncontrolled growth is checked, and the central nodule preserved intact, it increases in bulk and the cartilaginous structures appear. Development is slower than in the normal situation, and the gross structure is not always perfectly regular, but histologically, differentiation is normal. It does not, however, proceed far, and bone or even hypertrophied cartilage very seldom appears. On the other hand, when the embryonic thigh bones or femora from older embryos (5½ to 6 days' incubation) were grown in the same way they remained surprisingly normal even in anatomical appearance; they developed a typical periosteum, and formed periosteal bone. This presupposes the existence of a phosphatase, and on analysis of the tissues this enzyme was found to have been produced during the cultivation in vitro since it was not detectable in the original implant. The phosphatase in normal bone formation is associated with osteoblasts and hypertrophied cartilage cells, and this seems to be the case also in vitro, since the limb-buds from the earlier embryos only developed small-celled cartilage and showed no phosphatase activity. The periosteum of six-day embryos can form bone in vitro independently of the presence of the cartilage. Many types of tissue have been grown in which enzymes are active, but this is one of the few, if not the only case in which the enzyme can be clearly shown to be developed by the cells while in an artificial medium.

In contrast to the behaviour of the femur and limb rudiments, there is the behaviour of the cartilage of the jaw or Meckel's cartilage,[81] only part of which ossifies in the normal bird. Cultures from the part which does not

ossify may be grown in vitro for long periods and they continue to grow as small-celled cartilage, never developing any phosphatase, hypertrophying, or showing any osteoid tissue. The mesodermal tissue surrounding the cartilage, however, which in the animal will form membrane bone by direct deposition of osteoid substance, behaves similarly in vitro. Centres of ossification start in the culture and phosphatase has been shown to be present.

These experiments with the development of bone form clear examples of the difference between histo- and chemo-differentiation, and emphasize the independence of the two processes. There is no visible difference between the small-celled cartilage of the limb and that of Meckel's cartilage, yet one later develops phosphatase and the other does not. Of course this subsequent development of phosphatase may depend on the presence of other factors, for the early limb-bud cartilage very seldom hypertrophies or produces phosphatase when explanted in vitro. This indicates that between the third and sixth day of development of the normal limb-bud something happens to the cartilage cells, which causes them to be capable of this further chemodifferentiation, thus apparently, though not actually, reversing what perhaps must be considered the normal process of chemodifferentiation preceding histodifferentiation. But there are obviously stages in the process of chemodifferentiation. Whatever the change is that occurs to the cells, it has so far only very exceptionally been produced in vitro, at any rate with any certainty, and does not occur in Meckel's cartilage even in the normal situation.

The other point which the work on limb-buds, in common with work on other organ rudiments, shows, is the extraordinary power of embryonic cells for self-differentiation; that is to say, of producing organs and structures in an histologically normal manner when cut off from the rest of the body. Further discussion of this, however, must at the moment be deferred, and first the behaviour of the cartilage and bone cells under certain

other conditions in vitro must be considered. Periosteal tissue was among the first to be cultured in vitro, and in media of plasma and embryo extract it shows a very extensive migration of fibroblast-like cells, visibly almost if not quite indistinguishable from ordinary fibroblasts. Sometimes, particularly near the central implant, the cytoplasm has that granular appearance which characterizes osteoblasts in vivo, but this is not always obvious. Cells which grow out from cartilage itself in vitro are small and round. The cells from the perichondrium generally grow like fibroblasts and differ in appearance but little if at all from the cells of periosteal origin (often called osteoblasts), or from heart fibroblasts, muscle fibroblasts, or any other fibroblasts. They could all be classed as primitive mesenchyme cells, but it is now known that there are actually differences between these cell types which may be detected by indirect methods and which make it almost certain that the cells are in reality chemodifferentiated. Interesting methods for distinguishing the various types are by their relative growth rates [171] and their reaction to treatment with different concentrations of embryo extract.[170] ' Osteoblasts,' or periosteal fibroblasts (from periosteum) and ' chondroblasts ' or perichondral fibroblasts (from perichondrium) both behave rather similarly. A very small quantity of extract is sufficient to cause marked stimulation of growth, but if the extract forms too large a proportion of the medium (i.e., if there is more than 15 per cent of the original embryo juice), growth is retarded, and the cells may even show signs of degeneration. Muscle and heart fibroblasts, on the other hand, are much less sensitive to high concentrations of extract, in fact heart fibroblasts will show very little growth unless the concentration is over ten per cent. Correlated with this, the residual growth energy of ' osteoblasts ' and ' chondroblasts ' is greater than that of heart fibroblasts, that is to say they are capable of growing more freely and for a longer time in a non-growth-promoting medium like plasma. Perhaps they contain a higher concentration of the hypothetical substance which is re-

sponsible for the growth-promoting activity of embryo extract.

This similarity in behaviour of 'osteoblasts' and 'chondroblasts' with respect to the concentration of extract in the medium is interesting, since there is other evidence that these cells, although usually easily separable, are really not fundamentally very different. Both, for example, are capable of producing phosphatase, and the following experiments also illustrate the close connection between these cell types. It has been found that when cultures of periosteal bone [78] from the shafts of long bones are carefully prepared, so that only bone is present, the cells wander out from the substance of the bone and proliferate freely. If the central implant is then removed the cavity will become filled up with fresh tissue and within that new tissue bony substance is generally formed. In some instances, however, not only bone but cartilage also may develop. It is just possible that the cells contained within the Haversian spaces or canals in the bone may be cartilage cells as well as bone cells, but it is probably more likely that the osteoblasts from the bone are really capable of producing the same ground substance as cartilage cells when they meet the appropriate conditions. It is therefore conceivable that cartilage cells may show several stages in their differentiation. They may be able to grow rapidly like fibroblasts and deposit no ground substance, or perhaps a fibrous network. They may exist as small-celled cartilage which shows no evidence of changing in the direction of bone, and contains no phosphatase. Thirdly, hypertrophic cells may make their appearance and these contain phosphatase. The distinction between such cells and true bone cells appears to be a very subtle one. Although at least three of these types of behaviour have been produced in vitro, the actual factors which govern their appearance are still subjects for speculation and experiment. Some are undoubtedly intrinsic or perhaps more closely connected with the condition of the cell in the actual animal, as is made clear by the comparative inability of cartilage cells

derived from the very young limb-buds or from normally non-ossifying structures to develop the hypertrophic type of cell with its concomitant enzyme. Others probably depend on the conditions of culture.

(*c*) *Pure Strains of Cells*

Considerations such as the above throw some doubt on the validity of the conception of there being pure strains of cells in vitro,[30] in the same way as there are among bacterial cultures. Is it justifiable to conclude that a pure strain of osteoblasts has been produced, if suddenly some of the cultures start to produce cartilage ? The cells in tissue cultures are far more dependent on one another than are bacteria, and this interdependence persists. Among races of bacteria there is generally competition, and one race in the long run overgrows all the others. To some extent this is true with animal cells ; the fibroblast type of cell, for example, generally overgrows the epithelial type in conditions where uncontrolled growth is favoured more than growth with differentiation, and on these grounds it was supposed that eventually a pure culture of fibroblasts would be obtained. But quite obviously a pure culture of fibroblasts may mean very little, since there are very inadequate means of distinguishing between the different types of fibroblasts. By using different concentrations of embryo extract probably a certain but very limited separation can be obtained. For the culture to be considered as a pure culture it would seem reasonable to suppose that the cells composing it should all have about the same rate of growth ; in fact, this would form a standard for uniformity ; and if this were so the times between successive divisions might be expected to remain constant, but it has already been shown that there is the same variation in intermitotic period in a pure strain of fibroblasts twenty or more years old as there is in a culture made from fresh embryonic heart tissue. It is not only the rapidly growing cells which move to the outside of a colony, and many cells which do not divide at all may find their way there, so that no

separation is necessarily effected by always sub-culturing from one particular part of the culture, from the periphery for example. Except in cases of widely different types of cells like fibroblasts and epithelial cells, the method of obtaining pure cultures by the method of overgrowth is, to say the least of it, doubtful. A strain of cells perhaps grows rather more evenly and readily after it has been sub-cultured two or three times, but beyond that there is probably little to be gained by prolonged sub-culturing. The end-result is still, for all that is known to the contrary, a mixture of cells. It is certainly a mixture so far as growth rate is concerned, and as there are only very inadequate means of distinguishing types of cells otherwise it seems a little rash to regard them as all of one type, rather than as a mixture of several types growing harmoniously together. The fact that it has proved to be impossible to raise a strain of fibroblasts from a single cell, in so far as it can be used as an argument at all, argues in favour of cells in tissue culture being subservient to one another. At the present time the only justifiable method of establishing a pure strain would be to initiate it from cells all of one type, and the difficulties in this are enormous. The pigment cells of the retina would be a hopeful source because here there is a definite criterion of purity, since the cells readily develop pigment in vitro[175] and thus afford a visible means of distinction from cells of other types; but even this is not quite certain since wandering cells have been shown to be able to take up pigment granules when these are liberated into the medium by the breakdown of their containing cells.[176]

(*d*) *Differentiation in the Very Young Embryo*

Tissue culture affords an excellent method of investigating the capabilities of different types of tissue to develop, and as foreshadowed in what has gone before, it has been used extensively in experimental embryology, and recently a technique has been evolved for experiments on the potencies and capabilities of the tissues of very young embryos.

It has been found possible to culture the entire blastoderms of chick, duck and rabbit embryos[185] on the surface of plasma and embryo-extract coagula in moist chambers. The cultures do not persist for very long, but in these very early stages differentiation and the organization of the embryo occur with extreme rapidity, so that much happens in the space of a few hours. During the time in which the embryos survive they undergo perfectly normal growth and differentiation, except that the whole process is somewhat slowed down, particularly the growth rate, and there may also be minor defects in the gross anatomy, owing to the abnormal mechanical conditions which prevail. Differentiation is checked less than growth, so that compared with normal embryos these ectopic individuals are smaller but nearly as far advanced in structure. This is a general characteristic of organ rudiments developing in vitro and occurs in the development of later stages as well as in the very embryonic.

Not only can whole blastoderms be grown in this way, but they may be subjected to various experimental procedures. Before any actual organ rudiments are developed, and while the tissue which will form the embryo is still in the undifferentiated condition known as the ' primitive streak ', it may be divided up by transverse cuts and the parts separately cultured and their developmental capabilities analysed. Experiments of this character show that already the future embryo is foreshadowed. The cells of the anterior region near what is known as the ' primitive pit ' are those concerned with head formation, and the various organ-forming substances or regions are located down the body. Removal of the anterior region by a section through the ' primitive pit ' allows the posterior portion to develop much as it would have done in the uninjured blastoderm except that there is a tendency for the two sides to diverge. For example, a heart rudiment develops separately on each side. The part anterior to the cut develops practically only neural tissue, if it survives at all. Expressed differently, it might be said that there is a gradient of

organ-forming capacity down the body, and this is also borne out further by grafting experiments, in which there is shown to be once again very clear evidence for the influence of one type of tissue on the behaviour of another. In the early embryo it is possible to distinguish and separate two primary layers of cells, an upper layer or epiblast, which normally gives rise to neural tissue and epidermal structures, and a lower layer or endoderm, from which will arise the gut and related structures. The third primary germ layer or mesoderm is not distinguishable at this stage, and normally arises later chiefly from invagination of epiblast cells. If the epiblast is separated from the endoderm, and the two layers are rotated on each other, the primitive streak develops in the epiblast and the direction which it takes up is found to be dependent partially on the axis of the endoderm, that is to say that the primitive streak tends to have its anterior end bent towards the anterior end of the endoderm. But perhaps more interesting than this are the results of certain experiments in which parts of the primitive streak of one embryo were grafted between the epiblast and the endoderm of another embryo. In this situation the grafts survived and underwent some degree of development according to their origin, and they also influenced the behaviour of the epiblast of the host; moreover, the extent and nature of this influence depended on the portion of the primitive streak which was used. Not only is there a gradient of powers of development down the long axis of an early embryo, but the various isolated regions are capable of inducing behaviour in another epiblast corresponding to their potencies. The anterior two-thirds of the embryo are found to induce the development of neural tissue in the host epiblast, while the posterior third, which itself never produces neural tissue of its own, is incapable of inducing it in the host. The mechanism of this induction is presumably a similar phenomenon to that which has been found to occur in the development of certain amphibia, and to which brief reference has already been made. That is to say, the immediate proximity of cells of one

type so influences the behaviour of the cells of another type as to cause them to develop along certain definite lines, which perhaps they would otherwise not have taken, and this principle is a key to the behaviour of many types of embryonic tissue, but the greater part still remains to be unlocked. The influence is presumably of a physico-chemical nature and depends on the close propinquity of the cells. It is not species specific, and the interesting observation has been made that the influence can be exerted by dead cells, even cells which have been thoroughly coagulated by heat.[187] Parallel work being performed on the embryos of certain newts [167] shows that a substance may be extracted from their tissues by ether which will cause the formation of nervous tissue when embedded under the epiblast of developing gastrulae. In that case the 'organizer' has a very wide distribution, and is a substance which can be extracted from practically any differentiated tissue. There is some evidence, too, that the carcinogenetic polycyclic hydrocarbons are capable of effecting inductions in embryonic tissue. These observations throw quite a new light on the mechanism of the differentiation process. The 'organizer' might then appear to be something which was not only responsible for neural tube formation but for differentiation in general, and the neural tube merely took a prominent position owing to the fact that it develops first, and later-developing structures might be overlooked either because the tissue had been fixed too soon or because the whole preparation was not sufficiently healthy to continue normal development. In many cases there is evidence that the formation of an induced neural plate is in practice followed by the formation of mesoderm, &c., so that actual double monsters can be formed by these experimental methods. The induction of a gut frequently follows neural plate formation, but it is doubtful if a gut is ever formed when a neural plate is not; this might suggest that the tissue once induced then acted on others around it in such a way as to attempt to produce a whole embryo. That is to say that an induced neural tube behaves

towards, and organizes, the tissue round it in the same way that a normal neural tube would behave. The induced neural tissue becomes itself an ' organizer '. In this connexion it is important to notice that the ' organizer ' not only induces neural tissue, but a neural plate, that is nervous tissue organized and arranged in a way precisely similar to the normal arrangement. Possibly we have to deal with two distinct processes, evocation, or the calling into existence of the tissue, and individuation, or the organization of the evoked tissue in a ' normal ' manner. In practice the two processes may not be separable. With regard to evocation it is now commonplace but still fundamentally important that up to a certain stage of development practically any tissue may be susceptible, and moreover the same tissue may be susceptible in different ways, according to the nature of the graft. With regard to individuation, the process is considerably more complicated, and depends not only on the tissue itself but also on the position which it occupies in reference to the host tissue. The whole embryo is built on a system of gradients of developmental power, antero-posteriorly and laterally, so that when tissue is grafted between the epiblast and endoderm and induces the formation of structures in the epiblast, those induced structures are under the influence not only of the grafted tissue but also of the gradients of the host. The consequence is that the way in which the tissue finally develops is a compromise or resultant between the various forces. The nearer the induced tissue is to the actual host embryo the greater is the tendency for the new tissue to be incorporated in that of the host, and in any case the greater is the tendency of the new tissue to follow the orientation of the host. When the graft is near the periphery of the blastoderm and far removed from the host embryo the influence of the latter is less, and then the orientation of the induced tissue is much more dependent on the orientation of the graft.

A subject which has been brought into prominence recently in the study of embryonic growth, and which is

Fig. 9—Induction of neural tissue in birds. Two blastoderms of the chick grafted together

u.n.g., normal neural plate of upper blastoderm; **i.n.g.**, secondary induced neural plate in upper blastoderm, formed in relation to **l.n.g.** normal neural plate in lower blastoderm (*From Waddington, 'Phil. Trans. Roy. Soc.'*)

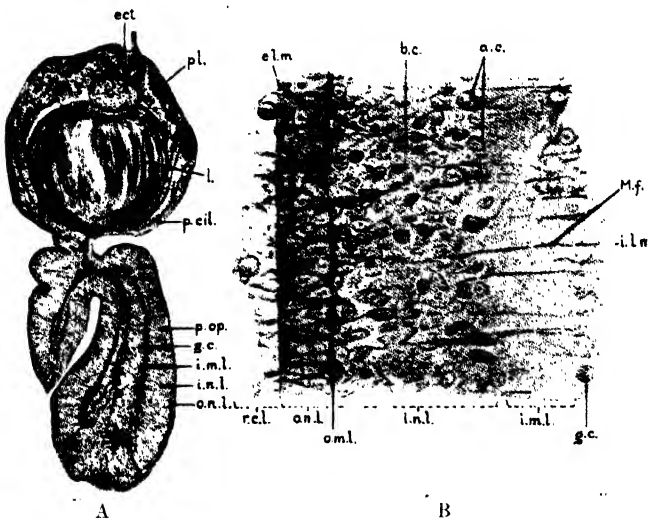

Fig. 10.—Development of the chick eye in vitro

A, section of an explant after eight days' cultivation in vitro; **B**, section through the retinal portion of an explant after seventeen days in vitro; **a.c.**, amacrine cells; **b.c.**, bipolar cells; **ect.**, ectoderm; **e.l.m.**, external limiting membrane; **g.c.**, ganglion cell; **i.l.m.**, internal limiting membrane; **i.m.l.**, inner molecular layer; **i.n.l.**, inner nuclear layer; **l.**, lens; **M.f.**, Muller's fibres; **o.m.l.**, outer molecular layer; **o.n.l.**, outer nuclear layer; **p.cil.**, pars ciliaris retinae; **p.l.**, pigment layer; **p.op.**, pars optica retinae (*From Strangeways and Fell, 'Proc. Roy. Soc.'*)

particularly well shown in ' quick-motion ' films of developing organisms, is the importance of tissue movements in the laying out of the tissues.[186] For example, in the formation of the primitive streak, there is a streaming of cells down the circumference of the blastoderm towards the posterior end, and when these streams meet they advance together up towards the centre. It has been described as a ' polonaise ' type of movement. The implications of these movements have not yet been worked out, nor indeed have their causes, but their regularity is certainly indicative of their dependence on definite gradients of one sort or another. That the epiblast cells are dependent on those of the endoderm during the movements which give rise to the primitive streak is made clear by the experiments already mentioned in which the two layers have been separated and then placed with a different orientation with respect to each other, and when it was found that the subsequent primitive streak was bent away from its normal direction towards the anterior end of the endoderm. Further experiments in which the endoderm has been completely reversed with respect to the epiblast indicate that the endoderm acts by initiating and influencing the movements of the epiblast cells rather than by any direct modification of their character. In these experiments the abnormal orientation of the endoderm has sometimes caused the length of the original primitive streak to be greatly shortened, and sometimes an entirely new primitive streak has been induced, and in a manner which indicates that a new set of tissue movements has been initiated. When a new primitive streak has been thus induced it may subsequently degenerate, it may persist along with the original one, or it may persist to the exclusion of the original. The result would seem to justify the idea that the endoderm acts on the epiblast to induce in the latter form-building movements of cells, and by altering the relative positions of the two layers at the appropriate times abnormal movements may be set up in such a way as to interfere with, compete with, or even reverse those which have already been started.

Presumably if the operation could be done early enough, reversal would ensue in every case.

Several facts of fundamental importance emerge from this work. In the first place the essential dependence of one group of developing cells on other groups in the vicinity is emphasized; this dependence is partly physico-chemical in character and may be partly mechanical. In the development of a normal animal there is started a continuous evolution in which there are several subsidiary and component processes which all work harmoniously together until, each part acting and reacting upon another, the animal takes shape as a whole. These processes include the establishment of axial gradients, the initiation of streaming movements, the evocation of special cell types, and their orientation and individuation. It now remains to work out the detailed mechanism of each process, and the successful experimentation on embryos in vitro, which has so recently begun, augurs well for the future.

No doubt these processes which are taking place on the grand scale in the early embryo continue with later development, but the mutual inter-relationships become more and more subtle and the influences of one cell type on another more delicate. In this way the organ rudiments appear to be what is known as self-differentiating, that is to say, that they need the influence of no outside factors to allow of normal development. Several beautiful examples of this self-differentiation have been followed out by experiments in vitro. The eye rudiment, for example,[182] has been removed from chick embryos of about sixty-six hours incubation and explanted on to the surface of semi-solid or solid coagula of plasma and embryo extract in tubes and transferred to fresh medium every two days. The technique is closely similar to the watch-glass technique already described. In such circumstances unorganized growth does not occur to any extent and the eye rudiment develops progressively. It increases in size, and histological examination shows that a very normal development has gone on within. From the cup of neural tissue, which in the embryo of the age

selected is surrounded by a layer of cells which may or may not contain pigment, and encloses a rudimentary group of cells which normally produces a lens in due course, there develop all the structures of a typical retina, pigment layer, nuclear and synaptic layers, even rods and cones, and all the structures connected with the pars ciliaris retinae and the lens. The eye is abnormal in shape, but then the mechanical conditions under which it has developed are completely different from those in the body and the influence of cells of other types in the

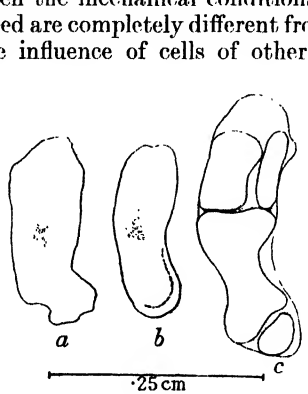

FIG. 11.—Camera lucida drawings of a living explanted blastema from a 2·18 mm. limb-bud, **a**, immediately after explantation; **b**, after 24 hours' cultivation; **c**, after 4 days' cultivation, showing definite separation of the bone rudiments and the formation of a joint. (From Fell and Canti.)

immediate vicinity has been removed. Moreover, the special food requirements of such a developing structure are not known, and it is significant that the size of the explanted eye is much less than that of control specimens in embryos of corresponding age. In other words, growth has been retarded by removing the tissue into artificial conditions, but differentiation has been allowed to proceed in a surprisingly normal manner. It is slowed down also, it is true, but less than the growth process. Exactly similar results have been obtained by cultivation of the otocyst rudiment in the same way.[77] Self-differentiation again occurs to a remarkable degree, with

the development of typical hair-cells, &c., but the whole structure is smaller than the normal and irregular in anatomy. The development of limb-buds, too, is interesting. If the original mesenchyme rudiment of the posterior limb-bud of a chick is excised and its development followed in vitro,[79] the cartilage develops first as a single unit, and then, later, changes take place within it which show that there are three distinct centres of activity, one corresponding to the femur, one to the tibia, and one to the fibula. The result is that even in the living preparation it is possible to distinguish the parts which would normally form the separate cartilages, and between them there are definite indications of the formation of a joint. After this stage is reached the limb is apparently no longer self-differentiating, for the distinction between the three rudiments disappears. Probably in the development of the limb in the whole animal other factors come into play about that time, not the least important of which may be the limb movements and the various mechanical stresses and strains which would result from them. In the formation of the three distinct units in these limb rudiment cultures there is again strong evidence for the importance of streaming movements of tissue, similar in character to those observed in the formation of the epiblast.

The meaning of self-differentiation as applied to these cases is probably little more than its meaning when applied to the whole embryo. Regarded as a whole, every embryo is self-differentiating, but in the experiments on early embryos it has been shown that in reality the development of each part is very largely, though not of course entirely, dependent on the behaviour of the parts around it, so that in the strict sense all development is probably dependent to a greater or less extent on factors external to the developing unit. It is the size of this unit which differs and gives rise to confusion. In the case of the limb-bud, by virtue of earlier tissue movements and orientations which are probably similar in character to those operating in the formation of neural tubes, &c., certain tissue develops which is determined

for limb formation. It is to some extent chemo-differentiated, and the process is irreversible, although susceptible to considerable modification. Once the limb-bud has reached this stage its further development becomes less dependent on its position in the animal, although even now such properties as right- or left-handedness are imposed upon it by the animal as a whole, and as already pointed out the complete development depends on the gross movements of the animal and its limbs. Meanwhile, there is within the limb-bud a mass of cellular tissue in which probably similar conditions are reigning as in an early embryo as a whole. Mass cellular movements, organizers and the influence of one cell on another, are probably the agents responsible for its development just as they are in mapping out the regions at an earlier stage in the entire embryo. Only the results of further experiment on isolating small fragments and studying their development and mutual interactions can say whether such a scheme is or is not a true picture of the developmental process, and unfortunately such work is fraught with great technical difficulties.

(*e*) *Blood, and the Nature of Wandering Cells*

But yet another interesting phenomenon has occurred in the tissue cultures from very young embryos. In the ordinary course of events in the development of the chick, part of the tissue first formed develops into embryo proper, and part develops into accessory membranes connected with the transference of food from the yolk sac to the embryo, with the respiration of the embryo, and with the elimination of its waste products. In connection particularly with the respiration is developed the area vasculosa and a very copious development of blood cells. Now it has been found that if portions of the early embryo in the primitive streak stage are transferred to hanging-drop cultures in plasma and embryo extract, or serum and embryo extract, there nearly always ensues in such cultures a great development of blood cells.[163, 164] Mostly erythrocytes are formed, but there is also evidence

for the appearance of granulocytes and endothelial cells and possibly of large lymphocytes. The red blood cells are formed in connexion with blood islands, and whereas the outer cells tend to become endothelial, the central cells of the islands become erythrocytes. Now tissue taken from different regions of the embryo and its membranes behaves very similarly in this respect when cultured in the manner described ; only tissue from the anterior quarter of the primitive streak failed to give any signs of blood formation. These results are extremely interesting in two ways. Firstly it is noteworthy that cells have the capacity for building haemoglobin under these artificial conditions of culture, which would seem to show that, given the right type of cell, haemoglobin does not require any special conditions for its formation. In this connexion it should be mentioned that previous cultures of haemopoietic organs [13, 76] have shown active formation of erythrocytes, but in them there is generally present a certain amount of haemoglobin in existing erythrocytes, which might be available to the young cells on the disintegration of their elders, a process which takes place readily in such cultures. The second point of great interest is why so much of the blastoderm is potentially blood-forming, whereas when treated in other ways it may behave quite differently. It has been suggested that here again the previous history of the cells has ' biased ' them in favour of blood formation or made them ' competent ' in this direction, so that in the absence of any further specific conditions they will self-differentiate into blood. The term ' biased ' is used rather than determined in order to indicate that the process is not irrevocable and that should suitable conditions arise, the cells might equally be capable of forming some other tissue, as indeed many of them probably would if left in the original embryo. Again a balance is struck between the inherent capabilities of the cell, which are themselves largely determined by the environment which that cell has encountered in the past, and the influences brought to bear upon it by the conditions under which the cell comes to be placed. The behaviour

of any cell then is conditioned partly by its intrinsic properties, which are in reality the impressions left upon it by all its previous experiences, which of course extend back not only to the immediate past but for generation after generation of its ancestors, and partly by its immediate environment. The impressions left by any set of conditions may be permanent and far-reaching, or slight and transient.

The problem of blood formation leads on to the very vexed question as to the relationships of the various amoeboid cells of the body. Under this heading are included the leucocytes or white cells of the blood, which may be divided into several groups by their characteristic appearances both in the living and in the fixed and stained condition, and the various wandering cells of the connective tissues, which include the group of actively phagocytic cells called by various names, macrophages, pyrrhol cells, reticulo-endothelial cells, stellate cells of Kupffer in the liver, histiocytes and so on. For reasons which will become obvious, the term wandering cell will be used here. In the blood may be distinguished cells with lobed nuclei and granules in the cytoplasm, collectively called granulocytes, and divided, according to the manner in which their granules stain, into three groups, eosinophils, basophils, and neutrophils or simply polymorphs. The last name refers to the shape of the nucleus. All these three types are actively amoeboid and phagocytic. Pseudopodia are extended and withdrawn in a manner essentially similar to the protozoon from which this type of motion gets its name. Granulocytes in the adult mammal are derived from the bone marrow in a manner similar to the erythrocytes, and the consensus of opinion is that they are effete cells with no future before them. Like erythrocytes they have embarked on a career from which there is no turning back, and death at the end of a few weeks seems to be their lot. In cultures of blood in various media, the granulocytes are active for a few days, but soon die and disintegrate without showing any signs of cell division.

The other main group of white cells in the blood consists of the lymphocytes and monocytes. The former have been divided into three categories, small, medium, and large, and the large lymphocytes are not easily distinguishable from the monocytes. All these cells have clear hyaline cytoplasm which stains with basic dyes and a single nucleus which in the larger types tends to be kidney-shaped. The small types have very little cytoplasm round the nucleus, the large lymphocytes have more, and the monocytes (also called macrocytes) have the most. In the monocytes, vital staining with neutral red typically shows a rosette of granules opposite the indentation of the nucleus. All these types are more or less motile, and the monocytes at least may have lamelliform pseudopodia. They all appear to be derived from the lymphoid tissue of the body, and their history and potentialities are still far from clear. The lymphoid tissue throws out daily into the general circulation enormous numbers of small lymphocytes,[205] derived seemingly by the division of cells resembling large lymphocytes situated in the germ centres of the lymph glands. What are they all doing and where are they all going?

The cultivation of these cells in vitro should provide a solution to the problem, but there are difficulties, not the least of which is the establishment of definite criteria by which to distinguish the various cell types. This has recently been very strongly emphasized by cultures of the 'buffy coat' of blood [5] in fluid media of various compositions. Blood was centrifuged so that all the corpuscles were separated from the plasma and in so doing the white cells settle on the surface of the red to form what is known as the buffy coat. These cells have been removed and cultured in a plasma coagulum for a few days in flasks and the plasma coagulum has been bathed with dilute serum. By this method monocyte-like cells creep out on the surface of the glass below the plasma coagulum, so that after a few days the plasma together with the remaining central mass of cells can be removed, leaving these monocyte-

like cells spread out and relatively isolated on the bottom of the flask in a manner peculiarly appropriate for observation and experimentation. There are differences of opinion as to the origin and nature of these cells, and also as to their prospective potencies, but one thing is now quite certain, and that is that their whole appearance and character is quite capable of changing with alterations in the constitution of the fluids bathing them. The cells may be round or spindle-shaped, or they may be granular or hyaline. Characters which would have satisfied the older histologists that the cells possessing them were quite certainly of different types can many of them now be produced at will in one and the same cell. No longer can cells be labelled this or that because they have a granule here or there. New criteria must be established and the difficulty lies in deciding upon what grounds these future distinctions shall be based.

In the particular case of the blood monocytes, if indeed they are all monocytes, cultivated in the manner outlined, when bathed with heparin plasma as fluid medium the cells are slender and elongated. In serum the cells become larger and filled with granules, and their whole appearance, rate of growth, and behaviour can be altered by bathing them with solutions containing digests of proteins, and tryptic digests behave differently from those effected by pepsin, producing in contrast to their action on fibroblasts more active growth. Breakdown products of proteins lead to the formation of overfed, round cells whose protoplasm becomes filled with fat globules and other cell inclusions, and the morphological differences can be partly correlated with the amino-nitrogen content of the medium. As in the case of fibroblasts pure amino-acid mixtures do not produce healthy growth, and all the products of digestion are in the long run ineffective without either plasma or serum. In tryptic digests there is a tendency for the cells to agglutinate. The acidity of the medium has been shown to be another factor which increases the granulation of the cells. All these con-

siderations point to the difficulty of establishing cell types on purely morphological grounds.

Although these cultures are made from the buffy coat of blood, and hence from a very mixed collection of cell types, it is believed that the cells which eventually survive are those which would be initially classed as monocytes. The small lymphocytes, granulocytes and erythrocytes all degenerate.[4] In the expanded and granular condition to which these monocytes come in culture they bear strong resemblance to the wandering cells of the tissues and it seems probable therefore that the wandering cells (histiocytes, macrophages, &c.) may be simply other functional states of the blood monocyte. Claims have been made that the wandering cells may transform readily into fibroblasts and vice versa,[17, 52, 53, 54, 90, 152, 154, 161] but there is still some doubt as to whether these claims can be substantiated and whether the apparent transformation is real. The wandering cell has several characteristics which would seem to establish it as a physiologically different cell type from the fibroblast,[44, 45] but if the fibroblast is not distinguishable from the primitive mesenchyme cell, then it is conceivable that wandering cells might be developed from such, and possibly might even revert to the same type. There is no doubt that under certain conditions wandering cells may look like fibroblasts, but such a criterion is quite obviously not sufficient. Wandering cells can grow and multiply on serum or plasma alone; they do not form a tissue, but grow as isolated cells, and colonies of such cells tend not to fuse but to remain apart. They often have pseudopodia in the form of undulating membranes, and, possibly correlated with this, a superficial nucleo-plasmic ratio in the living condition of 0·054 as opposed to 0·138 for fibroblasts. Such figures, however, in the light of what has been already said cannot be taken as very good evidence upon which to base a distinction[14, 178] but are merely corroborative. Certain differences, likewise, in the position and concentration of granules in the cytoplasm and shape of the mitochondria are also

fairly characteristic but again cannot in themselves be taken to indicate a definite difference. Fibroblasts on the other hand do not grow freely in plasma without the addition of embryo extract, and usually form connected colonies which will readily unite with each other. They do not liquefy the plasma coagulum as readily as wandering cells, and while the latter take up the vital dye trypan-blue and segregate it into large vacuoles, fibroblasts content themselves with a few minute droplets in the cytoplasm. Obviously then typical fibroblasts differ quite widely from typical wandering cells but this does not answer the question as to whether one type can under certain conditions become transformed into the other. Cases are described [90] in which suddenly a ' pure strain ' of fibroblasts will produce round the periphery of a culture a halo of cells identical in appearance with typical wandering cells ; but on those who would suggest that such cells have arisen from true fibroblasts lies the onus of proof that a direct transformation has occurred. Can they distinguish with certainty an undifferentiated mesenchyme cell from a fibroblast, so that they are sure that the wandering cells are not derived from such ? Can they be sure also that there have not remained wandering cells in the colony throughout its life, which, by some chance conditions of culture, have suddenly started active growth ? In other words, is it a true case of metaplasia, or is it a case of the delayed activity of one cell type ? Grounds have already been mentioned for believing that cultures of fibroblasts are anything but pure and at any rate at the start are always bound to contain a certain number of cells of other types including wandering cells. Similarly, occasions have been described where sheets of fibroblast-like cells occur in cultures of wandering cells,[52] but the evidence that such cells then have all the characteristic properties of fibroblasts is generally incomplete. The difficulties are two-fold. First the multiplicity of cell types which are habitually classed as fibroblasts and secondly the difficulty of following the history of indi-

vidual cells, and being able to assign them to any given type.

There is a school of workers who believe that in cultures from blood and from lymph and lymphoid tissue, the small lymphocytes do not degenerate,[16, 17, 152, 154] but by the acquisition of cytoplasm become transformed first into large lymphocytes, then into monocytes, histiocytes or wandering cells and finally even into fibroblasts. But again these results have been obtained by studying cultures from day to day and noting the dominant cell types present. No individual small lymphocyte has been followed right through the transformation, and large numbers of lymphocytes are observed to be in a degenerate condition. Moreover, in the cultures which were most successful, extraneous tissue in the form of fibroblasts was introduced.[17] If the possibility of contamination by these cells can be ruled out, an interesting problem is raised. Perhaps the lymphocytes depend for their well-being and development on the presence in their vicinity of fibroblasts, just as epithelia are apparently similarly dependent, so that the full values of cells, like those of colours, can only be brought out by the juxtaposition of other individuals of the right kind in their immediate vicinity. Further complications!

The theory of the embryonic character of the lymphocyte and the possibility of its transformation into monocyte, wandering cell and even fibroblast, is extremely attractive and backed by much circumstantial evidence. But much further research is necessary before it can be regarded as established, and meanwhile the fate of the vast numbers of lymphocytes produced daily by the animal body must be regarded as an unsolved problem.

(*f*) *Differentiation and Function*

So far have been considered chiefly those problems of differentiation which concern the establishment of the various cell types; a second aspect is also susceptible to study by tissue-culture methods, and that concerns the initiation of function by the differentiated cells.

In many cases cells may be unable to function properly under artificial conditions owing to the absence of some important constituent of the medium upon which the particular cells depend, and which they are unable to synthesize. The necessity for the presence of fibroblasts for the differentiation of epithelial cells may be a consequence of such a relationship. The metabolism of fibroblasts, being different from that of epithelial cells may lead to the production of substances, bi-products perhaps of their metabolism, which are nevertheless requisite for epithelial function. In other cases there may be no necessity for such a relationship and cells may take on their characteristic structure and function much as if still present in the intact organism. For example, the heart-muscle cell has been observed to acquire striations and to start contracting in vitro.[22, 96, 97, 142] Incidentally the cell may also show contraction without any evidence of striation. The latter cannot therefore be taken as a necessity for the contraction process of heart muscle, but presumably is an essential part of the make-up of the cell for the complete performance of its functions. In cells which are developing their striations there are first numerous bodies like mitochondria which arrange themselves in rows, and then become modified into the doubly-refracting rod-like elements which are an essential part of the structure which gives rise to the striations of muscles in general. Whether these bodies are mitochondria or not is uncertain but since normal mitochondria persist round the nucleus in an area of unspecialized cytoplasm it seems doubtful if they are. The independent contraction of heart-muscle cells in the entire absence of nervous tissue is perhaps the strongest evidence for the myogenic theory of the heart-beat, the theory that postulates that the rhythm of the heart is initiated by the muscle itself, and is only dependent on nerves for the control of its rate.

Ordinary skeletal muscle fibres[142] will maintain themselves for a few days in vitro and repair damaged surfaces, often growing out in bud-like processes from

their cut ends. Little, however, has been gained so far from their study in this manner which could not equally be obtained from a study of isolated fibres prepared simply by teasing.

With reference to plain muscle [142] there is no evidence in living cells for the presence of any fibrillae, but in

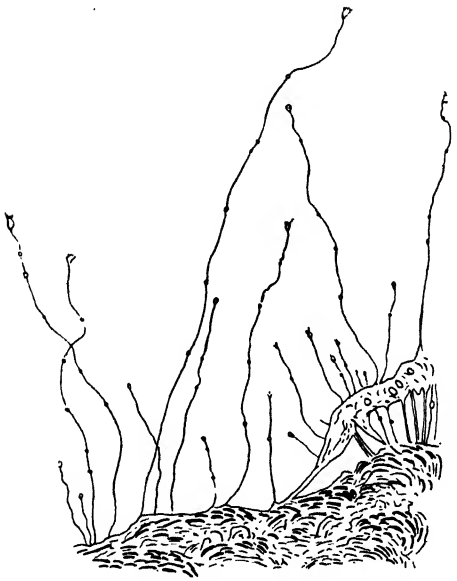

FIG. 12.—Diagram showing the mode of outgrowth of nerve-fibres from a fragment of chick brain

fixed cells the granules, &c., arrange themselves in such a way as to suggest the existence of tension striae, lying along the lines of force.

Nerve tissue was among the first types of tissue to be cultured in vitro, [103] and although it does not grow in the ordinary sense of cellular multiplication, yet the individual cells send out their processes into the medium often for very long distances. In a solid medium these

processes creep out from the central implant in more or less radial directions. [114, 115] Each process terminates in a definite end-knob, which is seen to be constantly sending out small amoeboid processes as though exploring the medium ahead of it. Along the length of the fibres, particularly towards their ends, can often be seen small swellings and varicosities of unknown significance.

The questions as to why nerves grow in certain directions, and as to how they reach their destinations in the developing body, have always aroused great interest. At one time it seemed possible [116] that the direction of outgrowth of cells in culture was susceptible to the influence of very weak electric fields, but the results were not substantiated. [195] It has on the other hand been known for some time that fibroblasts will follow lines of mechanical stress in a culture medium, [194] and more recent work [195] indicates that the movement of cells is largely controlled by the physico-chemical arrangement of the medium, that is to say, by the orientation of the protein micelles, and that the direction of outgrowth follows lines of orientated particles. In so far as physical, mechanical, and electrical forces may alter this orientation, just so far are they capable of modifying the direction of outgrowth of cells and nerve fibres, but it is improbable that electric fields have any direct orientating effect on the cells themselves, but only through their action on the medium. The factors therefore which control the paths taken by nerves in developing animals are probably mechanical or physico-chemical, rather than electrical in character, and it is probable that neurobiotaxis depends on the orientation of protein particles in the vicinity of the terminal processes of the nerve cells. This orientation may be the resultant of a variety of forces, among which those produced by the presence of cells in altering the water-balance of the medium in their immediate vicinity, for example, may be extremely important. Outgrowing nerves then, on these lines, would among other things depend on the activity of the cells adjacent to

them. Are neuroglia cells not so much supporting, but rather orientating cells ?

It might have been expected that tissue culture would have provided an excellent means for studying glandular function, and the changes which go on as a cell secretes. The results so far in this direction have been disappointing; but this is chiefly owing to the fact that the differentiation of cells only takes place readily in the central implant of the culture, and hence is not easily seen while the culture is alive. Generally speaking a high degree of differentiation is seldom reached by the cells in the periphery, and it is only such cells which are in a state in which they can be easily examined. However, one or two specialized types of secretion have been closely observed, as for example the formation of pigment, both in the pigment cells of the retina [175] and in connective tissue cells of dark breeds of fowls. [123] These are two essentially different types of cell, one epithelial or neural, the other of connective tissue origin. In both cases the pigment appears in the form of granules or rods, and these are foreshadowed in the cytoplasm by colourless granules which can be stained with neutral red. It is clear therefore that the mitochondria which stain with Janus green B and not neutral red do not play any immediate part in the formation of the pigment. Neutral red granules are more associated with the Golgi network which has often been shown, as in the case of mucous cells, to be associated with the formation of secretion granules. In the connective tissue cells the colourless granules became converted directly into pigmented bodies, and often two or three united together in a row and so formed the more rod-shaped pigment bodies. Pigment rods and granules move about in such cells with a rapid jerky movement, very much faster than the movement of the mitochondria, and give the impression that the cytoplasm is very fluid. The activity of their movement is said to increase with the intensity of the light shining on the cell.

In the account of tissue culture which has been given,

an attempt has been made to correlate the numerous fragments of scattered data which have so far been obtained and to orientate them in such a way as to illustrate the more fundamental contributions which the method has made to physiological knowledge, and to illustrate the manner in which a change of outlook has already been brought about, or in which such a change is likely to be necessary in the future. Tissue culture is not easy in practice, since, up to the present time, the number of variables in any given experiment is so high, and while living cells are under investigation this difficulty is ever likely to be present, but definite progress has been made. Probably its greatest contribution to science so far lies not in any one particular discovery, but in the fact that the study of isolated cells and tissues in vitro has altered the conception of the cell. It is no longer simply a subdivision of living matter with blue-staining nucleus, and paler cytoplasm. It is a living and changing unit, constantly varying in character and composition in response to changes in the fluid bathing it or in the activity of the cells surrounding it. This conception, for which tissue culture can claim a large share of the credit, opens up new channels for investigation of growth, differentiation and function. Whether such a change of outlook would have occurred without the help of tissue culture methods, no one can tell, but it is impossible to deny that they have materially assisted in bringing it about. Both in growth and in differentiation the mutual dependence of the cells of higher animals is emphasized, and the idea of the cell as an individual and self-contained unit comparable with a protozoon is definitely becoming more and more unlikely. Among the main problems which remain for the future may be mentioned first the nature of the growth process, and the meaning of age in tissues and animals, and secondly the further elucidation of the mechanisms by which tissues acquire their characteristic properties and differentiate into functioning units. Tissue culture has made beginnings in both these directions. Isolated prospectors have

probed here and there, methods have been established, and, in the terms of the prospectus for a mining company, great developments are hoped for in the future.

GLOSSARY

Alveoli. The vesicles in glands which are surrounded by the secreting cells.

Amoeboid. Moving with a motion resembling that of the protozoan Amoeba.

Basophil. Staining with basic dyes, e.g. with methylene blue.

Blastoderm. Disk of embryonic cells resting on an undivided yolk mass. It becomes divided into three layers, epiblast (ectoderm), mesoblast (mesoderm), and hypoblast (endoderm).

Blastomere. The name given to the constituent cells of a segmenting egg.

Blastopore. 'The next stage in differentiation consists in the conversion of the ball of cells (the blastula) into a double-layered sac or gastrula, by means of the process of gastrulation. Owing to the large amount of yolk present in the amphibian egg, this process is not as simple as in other forms (such as Amphioxus) where gastrulation is a simple invagination of one side of the blastula into the other. In the amphibian, the same result is achieved by the spreading of the cells of the animal hemisphere and their downgrowth over those of the vegetative hemisphere, at the same time as they tuck in or invaginate and then extend forwards beneath the surface of the outer layer. This process of spreading and growing over (epiboly), and of tucking in (invagination), first takes place on the dorsal side of the embryo, in the region of the grey crescent, and gives rise to a lip known as the dorsal lip of the blastopore' (*The Elements of Experimental Embryology*, J. S. Huxley and G. R. de Beer, Cambridge, 1934).

Carcinogenetic. Producing cancer.

Cartilage. Gristle. In many cases the forerunner of bone.

Chromosomes. Nuclear bodies visible at mitosis. Physical basis for inheritance. Carriers of the genes.

Coagulum. The jelly-like substance formed when plasma clots.

Collagen fibres. The tough resistant fibres in connective tissue.

Cytoplasm. The non-nuclear portion of the protoplasm of the cell.

Endothelium. The pavement epithelium which lines the inner surface of blood-vessels.

Enzyme. Ferment, or organic catalyst, hastening the speed of reactions.

Erythrocytes. Red blood-cells.

Fibrinogen. Fibrinogen (soluble protein in blood-plasma), when acted upon by thrombin, becomes insoluble fibrin, and forms a coagulum.

Gastrula. Stage in development occurring after invagination of blastula. (*See* Blastopore.)

Glutathione. A tripeptide of glycine, glutamic acid, and cysteine. The cysteine contains the SH group, which may be readily oxidized.

Golgi Apparatus (body). A cell inclusion rendered visible by impregnation with osmic acid, or silver salts. Function obscure.

Granulocytes. White cells of the blood whose nuclei are lobed, and whose cytoplasm contains granules.

Haematological. Concerning blood.

Haemoglobin. Iron-containing, red pigment of blood.

Haemopoietic. Blood-forming.

Heparin. An extract of liver which has an extraordinary capacity for preventing the clotting of blood.

Hyaline. Clear, resembling glass.

Invaginate. Tuck, or fold, in or under.

Lamelliform. Flattened, plate-like.

Leucocyte. Colourless nucleated corpuscle in the blood. White cell.

Limb-bud. Preliminary embryonic outgrowth, which later forms a limb.

Lymph. The fluid which drains away from the tissues of the body. Tissue fluid.

Lymphocytes. White or colourless cells of the blood with oval or kidney-shaped nucleus and clear cytoplasm. Small lymphocytes have practically no cytoplasm.

Lymphoid Tissue. Tissue in the animal where lymphocytes and monocytes are manufactured. This occurs in the spleen, in special lymph glands in the axilla, groin, &c., in the mesentery, and in the alimentary canal wall.

Mallory's Stain. A triple stain for connective tissue. Acid fuchsin, aniline blue, and orange G.

Meckel's Cartilage. A cartilage of the lower jaw.

Mesenchyme. The embryonic tissue which gives rise to the supporting structures of the body, to muscles, connective tissue, bone, &c.

Metabolites. Products of the metabolism, or physiological activity of cells.

Metaplasia. The transformation of one cell type into another.

Micelle. See Protein Micelle.

Monocytes. Large white corpuscles (cells) in the blood with single kidney-shaped nucleus, clear basophil cytoplasm,

and a rosette of granules which become visible when stained with neutral red.

Neural Plate. Region of specialized ectodermal cells which will later form the nervous system of the animal.

Neurobiotaxis. The mechanism by which nerves take up their arrangement and distribution in the animal body.

Nitroprusside Reaction. A red coloration is obtained if the solution tested contains reduced sulphur (SH groups) when it is treated with a saturated solution of ammonium sulphate, a few drops of a solution of sodium nitroprusside, and ammonia.

Notochord. Gelatinous supporting rod-like structure, in vertebrates largely replaced and surrounded by the vertebral column.

Optic Vesicle. Outgrowth of the primitive neural tube, which later forms the retina, &c., of the eye.

Osteoblasts. Bone-forming cells. (See p. 76.)

Pannet and Compton's Solution.

Solution A	Per cent
NaCl	12·11
KCl	1·55
$CaCl_2$	0·77
$MgCl_2.6H_2O$	1·27

Solution B	
M/69 $NaH_2PO_4.H_2O$	5 c.c.
M/69 $Na_2HPO_4.12H_2O$	55 c.c.

Solution A 4 c.c., Solution B 6 c.c., distilled water 90 c.c.

Periosteal Tissue. The layer of tissue which immediately surrounds bone. It consists of osteoblasts and fibroblasts.

Petrie Dish. Small, flat, circular dish with a cover of similar shape, as used in bacteriology.

Phagocytic. Capable of ingesting solid particles, bacteria, &c.

Physiological Salt Solution. Solutions of the necessary ionic concentration and osmotic pressure to enable cells to survive in them without damage.

Plain Muscle. Involuntary muscle of the alimentary canal, &c. It shows no striations.

Plasma. The fluid portion of blood. Under certain conditions, e.g. on the addition of tissue extracts, it forms a more or less solid jelly or coagulum, i.e. it clots. The protein constituent fibrinogen becomes converted into insoluble fibrin.

Polycyclic Hydrocarbons. Tar extractives, some of which have been found to be capable of producing cancers after repeated application to the skin.

Primitive Pit. A depression in the anterior region of the primitive streak.

Primitive Streak. The region of cells in the blastoderm which will form the embryo proper, as opposed to the embryonic membranes.

Protein Micelle. An aggregate of protein molecules, generally bearing an electric charge and containing more or less adsorbed water.

Prothrombin. Precursor of thrombin. Prothrombin + cephalin + Ca salts form thrombin.

Pseudopodia. Temporary and mobile extensions of cytoplasm.

Retina. The light-sensitive region of the eye.

Ringer-Locke Solution.

NaCl	0·9	per cent
$CaCl_2$	0·025	,, ,,
KCl	0·042	,, ,,
$NaHCO_3$	0·02	,, ,,

Rods of the Retina. Light-sensitive elements in the eye, particularly those concerned with vision in dim light (as opposed to the cones).

Serum. The fluid exuded from a blood clot. Plasma from which the fibrinogen has been removed as fibrin.

Spindle. A double cone of specialized protoplasm visible during mitosis, connected with the distribution and behaviour of the chromosomes.

Striations. Alternate dark and light bands visible in skeletal and heart muscle fibres.

Thrombin. The substance responsible for the conversion of soluble fibrinogen of blood plasma, into the insoluble fibrin of the plasma coagulum.

Tyrode Solution.

NaCl	0·8	per cent
KCl	0·02	,, ,,
$CaCl_2$	0·02	,, ,,
$MgCl_2$	0·02	,, ,,
$NaHCO_3$	0·1	,, ,,
NaH_2PO_4	0·005	,, ,,
Glucose	0·1	,, ,,

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